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Award Number: W81XWH-07-1-0373

TITLE:
Histone Methylation and Epigenetic Silencing in Breast Cancer

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REPORT DATE:
February 2011

TYPE OF REPORT:
Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

X Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
28-feb-2011	Final	01 JUL 2007 - 31 jan 2011

4. TITLE AND SUBTITLE
Histone Methylation and Epigenetic Silencing in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0373

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jeffrey A. Simon
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Minnesota
Office of Sponsored Projects

Minneapolis, MN 55455-2070

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This research was to investigate the role of a chromatin-modifying enzyme, called EZH2, in breast cancer epigenetics and to develop strategies to identify chemical inhibitors of this enzyme. EZH2 is a histone methyltransferase which modifies lysine-27 of histone H3, an epigenetic mark linked to gene silencing and implicated in tumor suppressor silencing during breast cancer progression. Progress on this project included: 1) Identification of target genes directly silenced by EZH2 in breast cancer cells, 2) Mapping of EZH2 association within the chromatin of one such target gene, 3) Demonstration that a DNA-binding protein, called YY1, co-localizes at this target chromatin, and 4) Evidence that YY1 is needed for silencing and EZH2 chromatin binding at this target gene. These results suggest that YY1 binding sites help define response elements that recruit and mediate EZH2 silencing in breast cancer cells. The defined EZH2 response element was evaluated for use in developing a breast cancer cell-based bioassay to screen for EZH2 inhibitors. These inhibitors provide important drug compounds to test as part of emerging epigenetic therapies to combat cancer.

15. SUBJECT TERMS
cancer epigenetics, histone modification, transcriptional repression, chromatin, histone methyltransferase, Polycomb group

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	44	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction

This research project was to investigate a chromatin-modifying enzyme, called EZH2, which is implicated in epigenetic modifications that contribute to breast cancer progression (Bracken et al. 2003; Kleer et al. 2003; Raaphorst et al. 2003; Cha et al. 2005; Bachmann et al. 2006; Collett et al. 2006; Ding et al. 2006; Simon and Lange 2008). EZH2 is a histone methyltransferase that modifies histone H3 on lysine-27 (Cao et al. 2002; Kuzmichev et al. 2002), which is a chromatin modification deployed in gene silencing (Cao and Zhang 2004; Simon and Kingston 2009). The purposes of this research were to: 1) reveal basic mechanisms and consequences of EZH2 function in breast cancer cells and 2) develop a breast cancer cell line-based bioassay to screen for inhibitors of the EZH2 histone methyltransferase. This research entailed identification of individual target genes silenced by EZH2 in breast cancer cells, the mapping and characterization of DNA elements within these genes that can mediate EZH2 silencing, and tests on these EZH2 response elements to evaluate their use in engineering cell lines that enable identification of small molecule inhibitors of EZH2.

Body

The body of this Final Report is organized with respect to Tasks within the Statement of Work (SOW).

Task I-2A: To identify target genes that are silenced by EZH2 in breast cancer cell lines

The first step towards defining an EZH2-response element (EZRE) was to identify target genes that are silenced by EZH2 in breast cancer cells. As shown in Fig. 1, we identified two such target genes. These EZH2-responsive genes are CCND2, which encodes the cell cycle regulatory protein cyclin D2, and MYT1, which encodes a transcription factor. We selected CCND2 for further detailed analysis, including chromatin immuneprecipitation (chromatin IP) studies to determine if and where EZH2 binds within the regulatory DNA region of this target gene. One key goal of these chromatin IPs was to distinguish whether EZH2 is a direct or indirect regulator of the target gene. Indeed, these studies revealed that EZH2 associates with the promoter region and with an upstream region located from -1.6 to -3.3 kb relative to the CCND2 transcription start site (Fig. 2). These results imply that the CCND2 regulatory

region contains response elements that attract EZH2 to this target gene and mediate transcriptional silencing.

EZH2 is the catalytic subunit of Polycomb repressive complex 2 (PRC2), which methylates target gene chromatin on histone H3-K27 (Cao et al. 2002; Kuzmichev et al. 2002). In order for PRC2-mediated silencing to occur at the majority of its known chromatin targets, it must cooperate with a second silencing complex, called PRC1. Indeed, we found that a PRC1 subunit, BMI-1, shows coincident distribution with the PRC2 subunit (EZH2) on CCND2 regulatory DNA. Taken together, these results substantiated CCND2 as an appropriate model target gene to study in breast cancer cells since it attracts both major Polycomb silencing complexes. We then turned our attention to delimiting the DNA regions upstream of CCND2 that mediate EZH2 silencing.

Task II-1: To identify and test DNA fragments from EZH2 target genes that can mediate EZH2 silencing (EZH2 response elements)

Neither PRC2 nor PRC1 contain subunits that are sequence-specific DNA-binding proteins. Furthermore, little is known about the DNA elements in mammalian target genes that recruit PRC2 and PRC1. Similarly, knowledge about trans-acting factors and mechanisms that target PRC2 and PRC1 to their appropriate chromatin sites around the genome is only now beginning to emerge (Simon and Kingston 2009). Thus, a major goal was to identify response elements within the CCND2 target gene and the trans-acting factors, referred to here as "recruiters", that bind these elements and serve to attract EZH2 and its associated silencing machinery.

<u>Tests for EZH2 recruiters</u>

Based on the work of others using embryonic stem cells and muscle cells, the YY1 and OCT4 DNA-binding proteins had been suggested as potential EZH2 recruiters (Caretti et al. 2004; Squazzo et al. 2006; Endoh et al. 2008). To address if these proteins might function as EZH2 recruiters in breast cancer cells, we performed chromatin IPs to map their associations with CCND2 regulatory DNA. We found that both YY1 and OCT4 bind to the same regions of CCND2 as EZH2: the promoter region and a more upstream region encompassing from -1600 to -3300 relative to the transcription start site (Fig. 2). Thus, the CCND2 upstream region provided a starting point to search for EZH2 response elements, which may feature YY1 and/or OCT4 binding sites.

A major effort was then pursued to address the functional importance of YY1 and OCT4 in silencing CCND2 expression and in recruiting EZH2 to the CCND2 target gene in breast cancer cells. As described in the 2009 annual report, technical issues prevented us from assessing the functional role of OCT4 in CCND2 silencing in SKBR3 breast cancer cells. However, we succeeded in analyzing the functional role of YY1. Specifically, we found that YY1 knockdown by RNA interference (RNAi) causes loss of EZH2 (a PRC2 subunit) and BMI-1 (a PRC1 subunit) from the CCND2 target gene (Fig. 3A) and a concomitant robust desilencing of CCND2 (Fig. 3B).

These results suggested that the YY1 DNA-binding protein recognizes and binds CCND2 regulatory DNA and that this, in turn, helps recruit EZH2 to the target locus. If this is correct, then YY1 binding sites should contribute functionally to the CCND2 DNA fragments that mediate EZH2 silencing (EZH2 response elements). Indeed, we found that there are 3 predicted YY1 sites in the CCND2 promoter region (spanning +250 to -450) and another 6 YY1 sites in the CCND2 upstream region (encompassing -1600 to -3300). Locations of these predicted YY1 sites are represented by asterisks in Figure 2. Similar results implicating YY1 in Polycomb silencing of a *Hox* target gene in differentiating human ES cells have recently been reported (Woo et al. 2010).

Tests for EZH2 response elements

To physically define EZH2 response elements, we required a functional assay for EZH2 silencing in breast cancer cells. Towards this end, we generated a set of luciferase reporter constructs for use in transient transfection assays. These constructs contain portions of CCND2 regulatory DNA, encompassing promoter and/or upstream regions, that our chromatin IP assays (Fig. 2) showed are associated with YY1, EZH2, and BMI-1. Examples of reporter constructs are displayed in Fig. 4. Our initial approach was to co-transfect such reporters along with an EZH2 expression construct to test for reporter silencing due to increased EZH2 levels. This approach employed MCF10A mammary cells since endogenous levels of EZH2 are lower in MCF10A cells than in SKBR3 cells. However, as detailed in the 2010 annual report, this over-expression/reporter assay proved to be insufficiently robust, most likely due to the limited increases (2-fold or less) in EZH2 levels achieved by transfection.

Consequently, we deployed an alternative reporter assay that relies upon EZH2 knockdown rather than over-expression. This approach seemed more likely to yield a robust assay for EZH2-mediated silencing in breast cancer cells because: 1) we had already established RNAi

conditions that routinely produce 8- to 10-fold EZH2 depletion in SKBR3 cells and 2) a similar reporter transfection/knockdown assay succeeded in demonstrating reporter silencing by a *Hox* gene DNA fragment targeted by PRC2 and PRC1 in mesenchymal stem cells (Woo et al. 2010). We transfected SKBR3 cells with the luciferase reporter constructs, subjected them to RNAi treatment to deplete EZH2, and then assayed for increased luciferase levels as a readout for desilencing. Parallel transfected samples were treated with either non-targeted control (NT2) dsRNAs or EZH2 dsRNAs and luciferase levels were compared. As shown in Fig. 4A, reporter constructs bearing either the CCND2 promoter (left panel) or a CCND2 upstream fragment (right panel) produced significant desilencing in response to EZH2 knockdown. A negative control SV40 reporter construct that entirely lacks CCND2 regulatory DNA was tested in parallel. As expected, this control construct showed no change in luciferase levels upon EZH2 depletion (not shown).

Having established a functional reporter assay, we next extended the analysis to determine if the same CCND2 fragments that respond to EZH2 loss also show desilencing upon knockdown of YY1. Our chromatin IP results, showing that YY1 loss leads to EZH2 dissociation from CCND2 chromatin (Fig. 3A), suggested that loss of either repressor should trigger CCND2 reporter desilencing. Indeed, RNAi treatments to deplete YY1 led to increased luciferase expression from the CCND2 constructs similar to that seen with EZH2 depletion (Fig. 4B). These results indicated that CCND2 fragments bearing YY1 binding sites can mediate gene silencing that depends on both YY1 and EZH2. Taken together, our findings suggest that at least one mechanism for chromatin targeting of EZH2 in breast cancer cells is via the YY1 DNA-binding protein. In a final series of experiments, we used site-directed mutagenesis in an attempt to define which of the nine consensus YY1 sites in the CCND2 upstream region (Fig. 2) might be most critical for reporter silencing. These assays did not pinpoint one or two key sites, suggesting that there may be redundancy and/or a number of contributing silencing elements in this upstream region.

Evaluation of identified EZH2 response element for use in high-throughput screening

Although we succeeded at identifying an EZH2 response element that could silence a linked luciferase reporter, we became concerned about two drawbacks of our EZRE-luciferase constructs for use in high-throughput screening for EZH2 inhibitors. First, the degree of EZH2 silencing observed, 2-to-3 fold (Fig. 4A), was not as robust as desired for high-throughput screening. Signal-to-noise in such screens is of key importance and we were concerned that

this modest level of desilencing would not be optimal. Second, the dependence on a partner repressor, YY1, and the large size (several hundred bp) of the silencing fragments would enhance odds of isolating inhibitors of off-target or indirect repressors rather than inhibitors of EZH2. We surmised that a better strategy might be to circumvent the normal targeting mechanism and the need for EZREs entirely by forcing direct association of EZH2 with a luciferase reporter. A prevalent approach for accomplishing this would be to tether EZH2 to a reporter by direct fusion to a DNA-binding domain such as GAL4. Indeed, proof-of-principle that this approach can provide robust EZH2 silencing of a luciferase reporter in human cells has recently emerged (Hansen et al. 2008). These workers demonstrated that GAL4-EZH2 can produce 10-fold silencing of a reporter bearing a multimerized GAL4 binding site. This technical advance suggests a potentially superior strategy for implementing the high-throughput assay to identify EZH2 inhibitors. Indeed, we submitted an Idea Development Expansion proposal, currently under review, to retool our strategy for this screen based on an assay that exploits GAL4-EZH2 silencing. We have become convinced that this alternative approach is more likely to yield direct inhibitors of EZH2.

Discovery of a regulatory mechanism that controls EZH2 silencing via phosphorylation

Our focus has been on identifying EZH2 inhibitors that disrupt its intrinsic enzyme function. However, it is also important to consider other modes of potential EZH2 inhibition that may impact cellular mechanisms that extrinsically modulate EZH2. Thus, we were highly intrigued when a local colleague in our Cancer Center, Dr. Haojie Huang, informed us of their discovery that EZH2 is phosphorylated in mammalian cells by cyclin-dependent kinases (CDKs). This was a key finding, since control of EZH2 in proliferating versus differentiated cells is not yet understood in mechanistic terms. Dr. Huang had found that this EZH2 phosphorylation contributed to target gene silencing but lacked data to address an underlying mechanism. In view of our expertise with PRC2 enzyme studies, Dr. Huang invited our collaboration on efforts to determine the molecular consequences of this EZH2 phosphorylation. Thus, during the final project year, the Simon lab performed in vitro tests on whether this phosphorylation (at T350) impacts intrinsic EZH2 enyzme function. These biochemical assays, which appear in Fig. S4A and S4B of the resulting published work (Chen et al. 2010), demonstrated that EZH2 phosphorylation did not alter intrinsic PRC2 enzyme activity. These results prompted Dr. Huang's lab to redouble efforts to assess whether EZH2 phosphorylation promoted gene silencing by instead potentiating the recruitment of EZH2 to target sites in cells. Indeed, their

subsequent chromatin IPs confirmed that EZH2 phosphorylation impacts targeting. These results established a new mechanism of EZH2 control and it raises the possibility of down-regulating EZH2 in mammalian cells by targeting its phosphorylation site or by modulating CDKs. Indeed, Dr. Huang also found that the majority of EZH2 target genes desilenced by impairing the EZH2 CDK-phosphorylation site are also affected by treatment with the CDK inhibitor, roscovitine. The manuscript resulting from this collaborative work is attached.

Key Research Accomplishments

1) Identification of target genes that are directly silenced by EZH2 in breast cancer cells.

2) Mapping of EZH2, BMI-1, YY1, and OCT4 sites of association within the CCND2 target gene in breast cancer cells.

3) Demonstration that the transcription factor YY1 is required to recruit EZH2 to CCND2 target gene chromatin and for transcriptional silencing of CCND2 in breast cancer cells.

4) Establishment of a transfection/knockdown assay to test CCND2 regulatory DNA fragments for function as EZH2 response elements. Use of this assay to demonstrate that this regulatory DNA can confer modest (2-3 fold) EZH2-dependent silencing upon a linked luciferase reporter.

5) Discovery that EZH2 is modulated by cyclin-dependent kinases, via a mechanism that impacts EZH2 targeting to chromatin sites of action.

Bibliography of Publications and Meeting Abstracts/ Reportable Outcomes

1) Simon, J.A and Lange, C.A. (2008). Roles of the EZH2 histone methyltransferase in cancer epigenetics. ***Mutation Research*** 647, 21-29. In special issue on "Epigenetics of development and human disease."

2) Chen, S., Bohrer, L.R., Nair-Rai, A., Pan, Y., Gan, L., Zhou, X., Bagchi, A., Simon, J.A. and Huang, H. (2010). Cyclin-dependent kinases regulate epigenetic gene silencing through phosphorylation of EZH2. ***Nature Cell Biol.*** 12, 1108-1114.

3) Wang, L., Nair-Rai, A., Lange, C.A. and Simon, J.A. (2008). Analysis of EZH2 function and target gene silencing in breast cancer cells. Era of Hope Meeting, DOD Breast Cancer Research Program, Baltimore MD.

List of Personnel Receiving Pay from the Research Effort

1) Jeffrey A. Simon, Ph. D., Principal Investigator
2) Carol A. Lange, Ph.D., Co-investigator
3) Liangjun Wang, Ph.D., Senior Research Associate
4) Aswathy Nair-Rai, M.S., Graduate Research Assistant

Conclusion

This research identified individual EZH2 target genes in breast cancer cells. One of these target genes, CCND2, was exploited in further studies to identify and delimit DNA elements that can mediate gene silencing by EZH2. This research also provided insight to mechanisms that recruit EZH2 to target genes in breast cancer cells. Specifically, the zinc finger DNA-binding protein, YY1, was implicated in targeting EZH2 to the CCND2 target gene. The characterization of an EZH2 response element allowed us to evaluate its use in cell-based strategies for isolating small molecule inhibitors of the EZH2 chromatin-modifying enzyme. We concluded that circumventing the EZH2 targeting mechanism, by forcing direct association of a tethered EZH2 to a luciferase reporter, would likely provide a superior strategy for developing a high-throughput screen for EZH2 inhibitors. Such inhibitors provide important lead compounds for the development and optimization of potential therapeutics that block EZH2 function. These inhibitors, and their derivatives, may find use in emerging strategies to combat cancer progression via drugs that alter epigenetic states of genomes in cancer cells (Egger et al. 2004; Lyko and Brown 2005; Yoo and Jones 2006).

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Supporting Data

Figures 1-4, which accompany the Body of this FInal Report, are appended below.

Figure 1

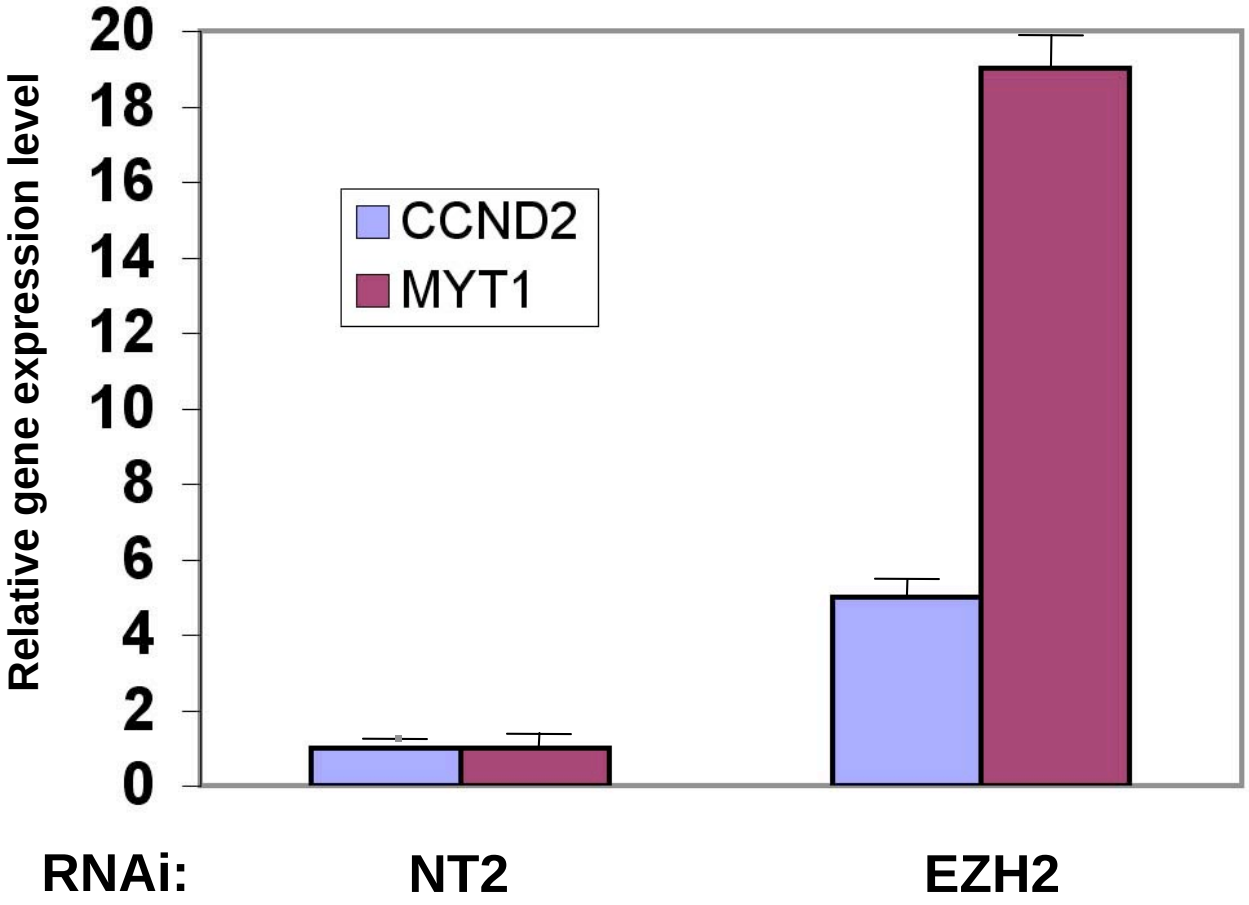

Figure 1. EZH2 knockdown derepresses the CCND2 and MYT1 target genes in breast cancer cells. Bar graphs depict relative abundance of CCND2 and MYT1 mRNAs as determined by quantitative (real-time) RT-PCR. RNA samples were prepared from SKBR3 cells treated with a pool of double-stranded oligonucleotides to deplete EZH2 or with a non-target (NT2) pool of oligonucleotides as negative control. Error bars represent standard deviations from triplicate samples.

Figure 2

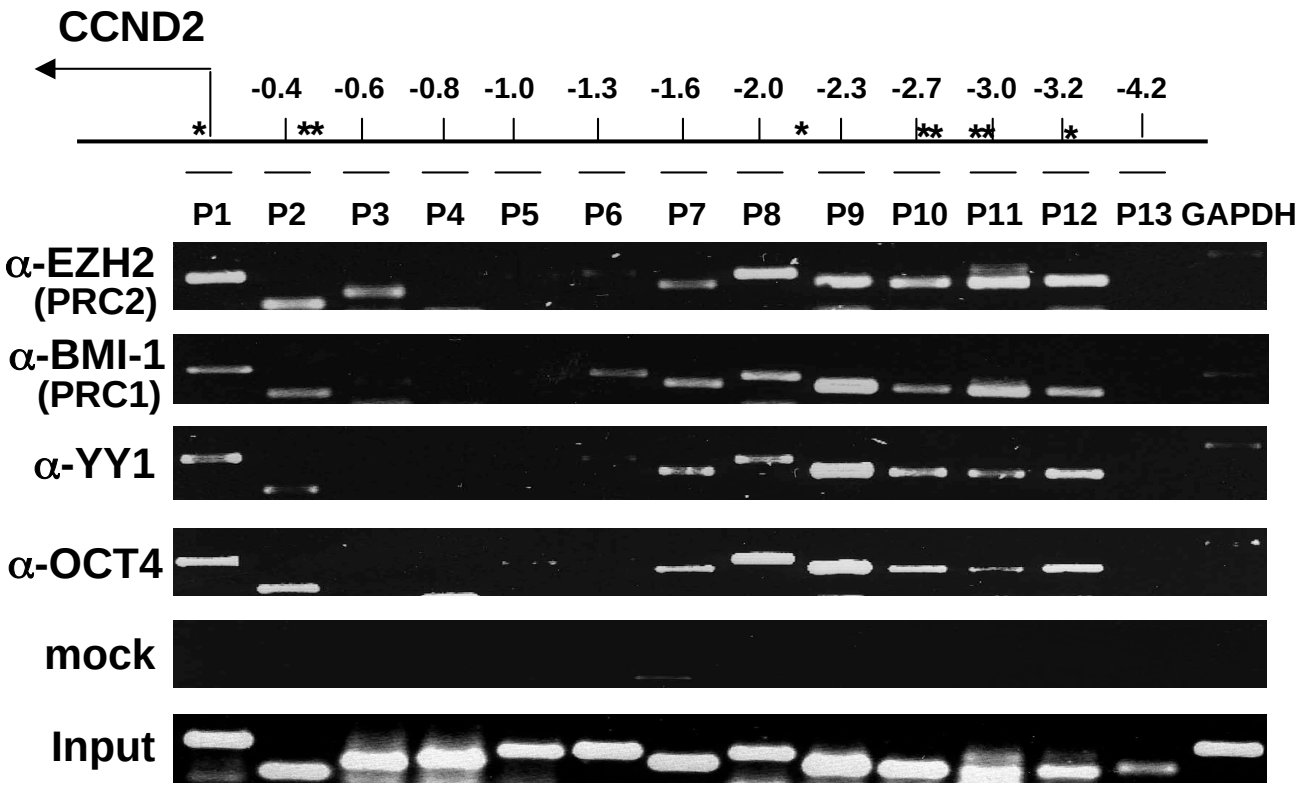

Figure 2. Mapping of EZH2, BMI-1, YY1, and OCT4 chromatin association within the CCND2 upstream region. Chromatin IPs were performed on SKBR3 cells, with successive primer pairs used to map indicated regulatory proteins within the 4 kb region upstream of the CCND2 start site (arrow). Top four panels show association of EZH2, BMI-1, YY1, and OCT4, as indicated. EZH2 is a subunit of PRC2 and BMI-1 is a subunit of PRC1. The fifth panel shows a mock IP negative control and the sixth panel shows PCR products amplified from input genomic DNA. GAPDH is a negative control gene which shows little or no association with these regulatory factors. Asterisks denote locations of predicted YY1 binding sites based on matches to consensus sequence.

Figure 3

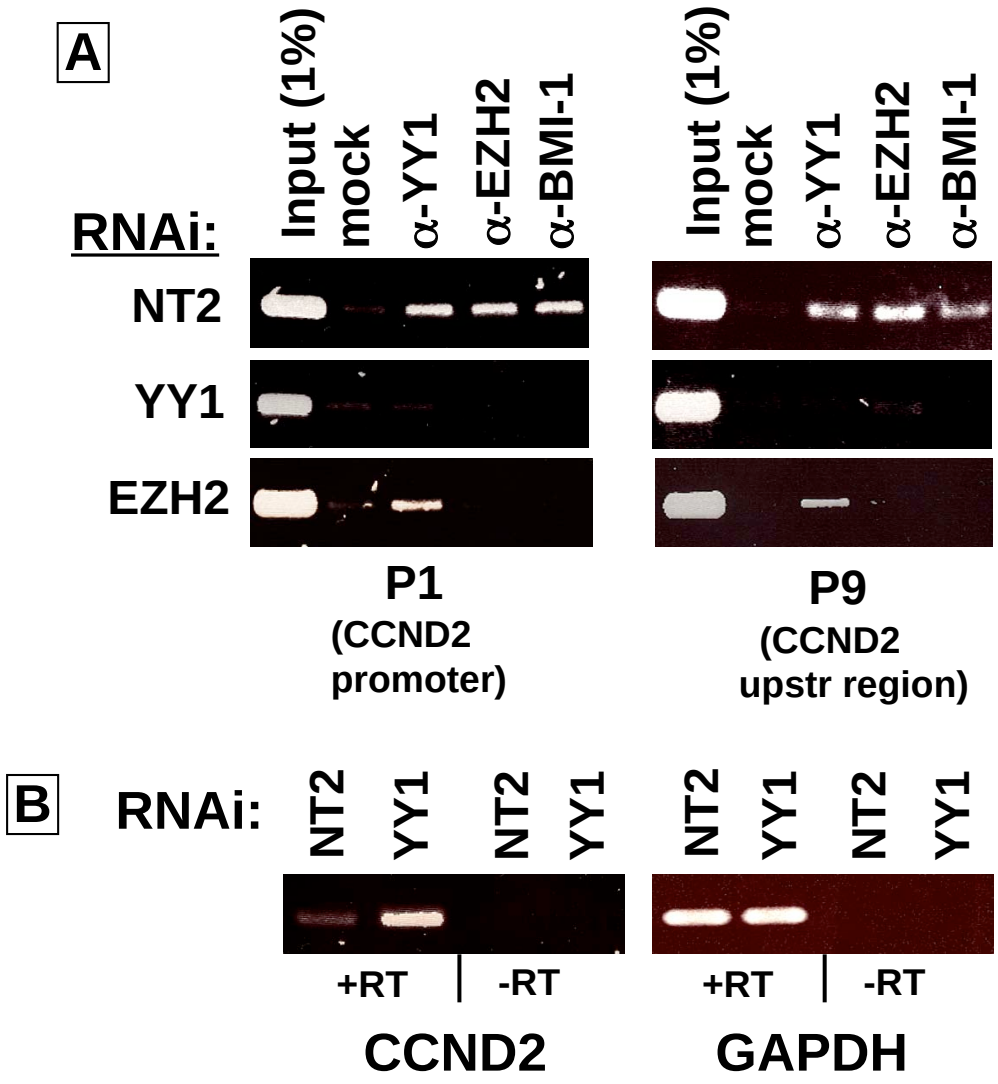

Figure 3. Role of YY1 in recruitment of chromatin factors to, and transcriptional silencing of, the CCND2 target gene. A) Chromatin IPs were performed on SKBR3 cells treated with either a non-targeted control pool of ds RNAs (NT2) or pools of dsRNAs to deplete YY1 or EZH2. Immuneprecipitations of cross-linked chromatin samples were performed using antibodies against YY1, EZH2, or BMI-1, as indicated. "Mock" indicates negative control immuneprecipitation lacking antibody. Protein associations with chromatin from the CCND2 promoter region (fragment P1, see Fig. 2) and CCND2 upstream region (fragment P9, see Fig. 2) were determined. B) RT-PCR analysis of *CCND2* and *GAPDH* mRNA expression after treatment of SKBR3 cells with a non-targeted (NT2) pool of control ds oligonucleotides or with a pool of ds oligonucleoticdes targeted against YY1. "+RT" indicates reverse transcriptase added and "-RT" indicates control with reverse transcriptase omitted. *CCND2* mRNA levels increase upon YY1 knock-down whereas control *GAPDH* mRNA levels are unchanged.

Figure 4

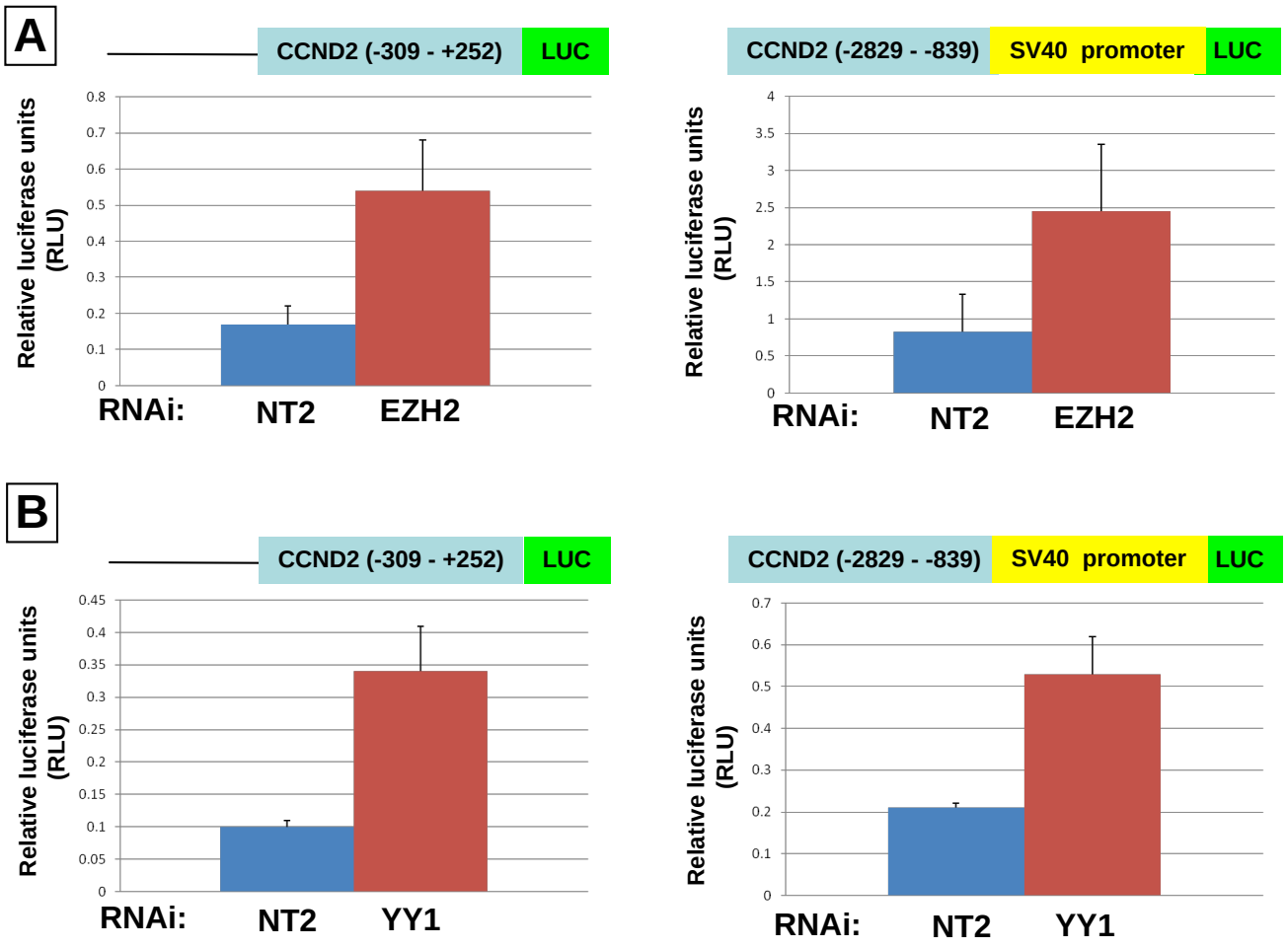

Figure 4. Reporter constructs and assays for EZH2 and YY1 silencing in breast cancer cells. Transfection constructs used in each assay are displayed above each bar graph. Constructs contain indicated CCND2 regulatory DNA fused to the coding region of the firefly luciferase gene. The construct shown in left panels contains CCND2 upstream DNA encompassing its own promoter whereas the construct in right panels bears CCND2 upstream DNA with the SV40 promoter. A) EZH2 knockdown: Blue bars show relative luciferase expression levels (firefly versus *Renilla* control) in SKBR3 cells treated with a control non-targeted dsRNA (NT2) and red bars depict relative luciferase levels in cells treated with EZH2 dsRNA. Reporter desilencing is observed upon EZH2 depletion. B) YY1 knockdown: Blue bars show relative luciferase expression levels (firefly versus *Renilla* control) in SKBR3 cells treated with a control non-targeted dsRNA (NT2) and red bars depict relative luciferase levels in cells treated with YY1 dsRNA. Reporter desilencing is observed upon YY1 depletion. In all graphs, error bars represent standard deviation derived from assays performed in triplicate.

Mutation Research 647 (2008) 21–29

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Review

Roles of the EZH2 histone methyltransferase in cancer epigenetics

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ARTICLE INFO

Article history:
Received 28 May 2008
Received in revised form 11 July 2008
Accepted 23 July 2008
Available online 3 August 2008

Keywords:
Chromatin
Cancer epigenetics
Gene silencing
Histone methyltransferase
Polycomb
EZH2

ABSTRACT

EZH2 is the catalytic subunit of Polycomb repressive complex 2 (PRC2), which is a highly conserved histone methyltransferase that targets lysine-27 of histone H3. This methylated H3-K27 chromatin mark is commonly associated with silencing of differentiation genes in organisms ranging from plants to flies to humans. Studies on human tumors show that EZH2 is frequently over-expressed in a wide variety of cancerous tissue types, including prostate and breast. Although the mechanistic contributions of EZH2 to cancer progression are not yet determined, functional links between EZH2-mediated histone methylation and DNA methylation suggest partnership with the gene silencing machinery implicated in tumor suppressor loss. Here we review the basic molecular biology of EZH2 and the findings that implicate EZH2 in different cancers. We also discuss EZH2 connections to other silencing enzymes, such as DNA methyltransferases and histone deacetylases, and we consider progress on deciphering mechanistic consequences of EZH2 overabundance and its potential roles in tumorigenesis. Finally, we review recent findings that link EZH2 roles in stem cells and cancer, and we consider prospects for integrating EZH2 blockade into strategies for developing epigenetic therapies.

Contents

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doi:10.1016/j.mrfmmm.2008.07.010

1. Introduction

Chromatin changes have long been associated with cancer (reviewed in [1–5]). The most well-characterized alteration is CpG DNA hypermethylation which often accumulates in promoter regions of tumor suppressor genes, thereby contributing to tumor suppressor loss through epigenetic silencing [1]. CpG hypermethylation at specific loci is so commonly observed in breast, prostate, and a wide variety of other cancers that this epigenetic alteration is considered a molecular marker of these diseases [6]. In addition to DNA methylation, epigenetic modification states of histones are also implicated in oncogenesis [2,7,8]. Particular global patterns of acetylation and methylation of histones H3 and H4 are associated with multiple cancer types and, in the case of prostate cancer, these modification patterns distinguish disease subtypes and can predict patient outcome [9,10]. These and other findings promote an emerging view that epigenetic changes in the cancer cell genome may contribute just as significantly to disease progression as do genetic alterations to DNA sequence [1,7,11]. However, there is a crucial difference between genetic and epigenetic alterations, which has important implications for development of cancer treatments. Once the DNA sequence is changed by mutation, it is difficult to restore the gene or counteract the altered gene product. However, epigenetic changes can potentially be reversed with inhibitors that block the relevant chromatin-modifying enzymes. Thus, it is important to identify mechanisms of epigenetic enzymes in cancer cells with an eventual goal of developing strategies to impede their undesired activities.

2. Molecular biology of the EZH2 histone methyltransferase

Among the histone modifications associated with gene silencing and cancer, much has been learned recently about the enzymes responsible for methylation of histone lysine residues [2,8,12]. Here we focus on one of these histone methyltransferases, called Polycomb repressive complex 2 (PRC2), which is the major enzyme that methylates lysine-27 of histone H3 (H3-K27). When PRC2 methylates this residue, it can add up to three methyl groups to the ε-amino group of the lysine side chain. The tri-methylated form of H3-K27 is currently viewed as the predominant form that conveys biological function in vivo. Thus, referral here to methyl-H3-K27 indicates the tri-methylated form, unless stated otherwise.

PRC2 was initially purified and characterized from human cells and *Drosophila* embryos [13–16]. As depicted in Fig. 1A, human and fly PRC2 have very similar core subunit compositions. They each contain a conserved catalytic subunit, EZH2 in humans or E(Z) in flies, which contains the signature SET domain that provides the methyltransferase active site [17]. Structure determinations of other SET domains have revealed an unusual "thread-the-needle" structure, called a pseudoknot ([18,19] for reviews). The pseudoknot is formed by juxtaposition of two conserved peptide motifs within the SET domain, with one peptide inserted through the loop created by the other. These structures show that the substrate lysine and methyl donor cofactor bind opposite sides of the SET domain with their binding pockets connected by an interior channel that aligns the reactive groups for methyl transfer. However, the EZH2/E(Z) subunit lacks enzyme function on its own. Instead, EZH2/E(Z) must be complexed with at least two of its noncatalytic partners, EED/ESC and SUZ12, to attain robust histone methyltransferase activity [20–24].

Fig. 1B displays the domain organization of EZH2. Both the C-terminal SET domain and the adjacent cysteine-rich CXC domain are required for histone methyltransferase activity [13–15,20]. As also indicated in Fig. 1B, additional N-terminal domains provide binding sites for assembly with the required partner subunits. PRC2 enzyme function can also be influenced by another associated component, called PHF1 in human cells and PCL in flies. Although PHF1/PCL is not a core subunit of PRC2 (Fig. 1A), its association with the complex can stimulate PRC2 enzyme activity and/or influence its recruitment to target genes in vivo [25–28]. The highly collaborative nature of the PRC2 enzyme complex, with multiple partners and inputs needed for function, is a key feature to consider in developing strategies for inhibition. That is, besides the catalytic site housed within the EZH2 SET domain, there are potentially many surfaces and binding pockets that could provide useful targets for binding inhibitory molecules.

A common biological function of PRC2 is transcriptional silencing of differentiation genes. Indeed, the role of PRC2 subunits in silencing *Drosophila* Hox transcription factors has long been recognized [29,30]. We now appreciate, through genome-wide studies (see below), that frequent PRC2 targets are transcription factors and signalling components with key roles in cell fate decisions in a wide variety of organisms. PRC2 and H3-K27 methylation are also implicated in mammalian X-chromosome inactivation [31] and the *C. elegans* version of PRC2 methylates H3-K27 and functions in

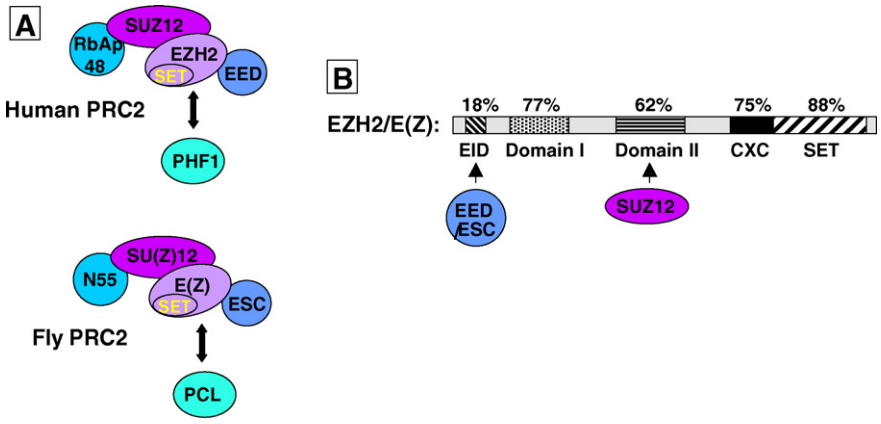

Fig. 1. Composition of PRC2 and domain organization of EZH2. (A) The four core subunits of human PRC2 are EZH2, EED, SUZ12 and RbAp48 [14,15] and the corresponding homologous subunits in fly PRC2 are E(Z), ESC, SU(Z)12 and NURF55 [13,16]. EZH2/E(Z) is the catalytic subunit that contains a SET domain. PHF1/PCL is another Polycomb protein that can associate with PRC2 to influence its activity and/or targeting [25–28]. (B) Five functional domains in EZH2/E(Z) are depicted, with % identities between the human and fly versions indicated. The SET domain houses the histone methyltransferase active site and the CXC domain also contributes to activity. Robust methyltransferase requires EZH2 assembly with both EED/ESC and SUZ12, and domains required for binding these noncatalytic subunits are indicated [20–22,114].

germline silencing [32]. Plant versions of PRC2 deposit K27 methylation to regulate key events in seed and flower development [33]. This remarkable conservation of PRC2, including its catalytic EZH2 subunit, indicates an ancient strategy for chromatin silencing that deploys H3-K27 methylation as a repressive mark. Although the underlying mechanisms remain to be determined, there is ample evidence that disruption of this epigenetic silencing system contributes to oncogenesis.

3. EZH2 overabundance in cancer tissues

Since the basic discovery that EZH2 functions as a chromatin-modifying enzyme, many reports have appeared that link EZH2 to the altered properties of cancer cells. The common finding is that EZH2 levels are abnormally elevated in cancer tissues versus corresponding normal tissues, with the highest EZH2 levels correlating with advanced stages of disease and poor prognosis. In some cases, EZH2 overabundance is paralleled by amplification of the EZH2 gene. Table 1 provides a compilation of studies that report EZH2 overabundance in tissue samples from patients with different types of cancer. Among these, altered EZH2 levels have been most extensively documented in prostate and breast cancer. The functional consequences of EZH2 over-expression, which may include hypersilencing of genes that promote differentiation and restrain proliferation, are discussed in Sections 4–6 below.

3.1. EZH2 in prostate cancer

One of the earliest reports was a gene profiling study where EZH2 was scored as the most significant gene up-regulated in metastatic prostate cancer compared to clinically localized prostate cancer [34]. This study also showed that loss of EZH2 inhibits growth of prostate cancer cells. Similar requirements for EZH2 in proliferation of other cell types have been described [35,36]. Significantly, EZH2 over-expression in prostate cell lines led to silencing of a discrete set of >100 target genes, which was dependent upon an intact SET domain [34]. Thus, this work suggested that EZH2 overabundance alters the genomic expression program through chromatin hypersilencing. Statistical analysis also revealed that EZH2 levels could provide a valuable prognostic indicator of patient outcome [34] and subsequent studies have described the prognostic value of combined sets of prostate markers that include EZH2 overabundance [37,38]. More recently, a Polycomb repression "signature", consisting of a cohort of 14 repressed EZH2 target genes, has been described as a tool for predicting prostate and breast cancer patient outcomes [39].

Table 1
Human cancers associated with over-expression of PRC2 subunits

PRC2 subunit	Type of cancer	References
EZH2	Prostate	[34,37,38,41,115,116]
	Breast	[35,40–43,117]
	Lymphoma	[118–120]
	Myeloma	[36]
	Bladder	[121–123]
	Colon	[124]
	Skin	[41,125]
	Liver	[126]
	Endometrial	[41]
	Lung	[127]
	Gastric	[128]
SUZ12	Colon	[45–47]
	Breast	[45,47]
	Liver	[47]

3.2. EZH2 in breast cancer

Analyses of patient samples significantly correlate abnormally elevated EZH2 levels with invasiveness and increased proliferation rates of breast carcinomas [35,40–42]. These studies also emphasize EZH2 as a prognostic indicator of outcome in breast cancer patients [40,42], reflecting the significant association of high EZH2 levels with aggressive forms of the disease. EZH2 accumulation may even provide an early molecular marker to detect precancerous changes in histologically normal mammary tissue [43]. To address cause-and-effect relationships between EZH2 function and oncogenesis, the consequences of engineered EZH2 over-expression in mammary cells have been examined. These studies have shown that EZH2 over-abundance in breast epithelial cells causes anchorage-independent growth and increased cell invasiveness in vitro [42] and EZH2 over-expressing cells are tumorigenic when injected into the mammary fat pads of nude mice [44]. Another study, using myeloma cells, showed that the oncogenic properties of EZH2 in mice correlate with its histone methyltransferase activity [36].

3.3. Cancer links to other PRC2 subunits

Less is known about cancer-associated alterations in PRC2 subunits besides EZH2. EED over-expression in human cancers has not been widely reported and the studies that first documented EZH2 over-expression in prostate and breast cancer revealed unchanged EED levels in the same patient samples [34,42]. However, elevated abundance of a particular EED isoform, EED2, has been described for breast and colon tumors ([45]; see below). There are also examples of SUZ12 alterations in cancer tissues (Table 1) including over-expression in colon, breast and liver tumors [45–47]. In addition, SUZ12 (also called JJAZ1) is implicated in endometrial cancer since a chromosome rearrangement creating a SUZ12 fusion protein is frequently associated with endometrial stromal tumors [48]. Finally, over-expression of PCL3, which is a homolog of the PRC2-associated protein, PHF1 (Fig. 1A), is also associated with many cancers including colon, skin, lung and liver [49]. Since consequences of EZH2 overabundance in cancer cells are still emerging, it is an open question if excessive levels of these noncatalytic partners work through similar mechanisms.

4. Collaboration of epigenetic silencing enzymes: functional links between EZH2 histone methyltransferase, DNA methyltransferases, and histone deacetylases

4.1. EZH2 links to DNA methylation

Polycomb silencing and DNA methylation have often been considered biochemically independent gene silencing systems. In agreement with this, *Drosophila* and *C. elegans* deploy PRC2 and H3-K27 methylation in silencing yet little or no DNA methylation is detected in their chromatin. However, recent studies in human cells, showing that EZH2 and DNA methyltransferases (DNMTs) are physically and functionally linked, have fundamentally altered this outlook and prompted important new models in cancer epigenetics. The key initial study [50] showed that PRC2 subunits (EZH2 and EED) co-immuneprecipitate with all three human DNMTs and that silencing of certain target genes requires both EZH2 and DNMTs. Significantly, RNAi knockdown in osteosarcoma cells showed that EZH2 is needed for DNMT binding and CpG methylation of target genes but, conversely, DNMTs are not needed for EZH2 chromatin association [50]. These data suggest a pathway where EZH2 acts upstream of DNMTs to methylate and silence target chromatin

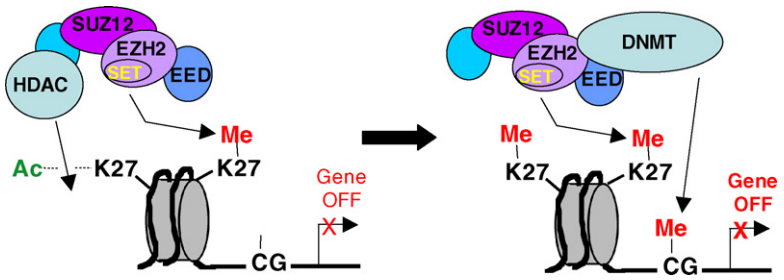

Fig. 2. Model for collaboration of epigenetic silencing enzymes. Target genes are initially silenced through histone H3-K27 methylation by PRC2. If K27 is pre-acetylated, then methylation of this residue may first require deacetylation by a histone deacetylase (HDAC), which are known to interact with PRC2 [14,57]. PRC2 may also recruit DNA methyltransferases (DNMTs) [50] which methylate CpG DNA of target genes, leading to a more permanently or deeply silenced chromatin state [52–54]. These chromatin modifications encompass many nucleosomes and CpG elements per target gene; for simplicity, only a single nucleosome and CpG element in an upstream regulatory region are shown. "Ac" denotes acetylation and "Me" denotes methylation.

(Fig. 2). It is not yet clear if DNMTs are recruited primarily by direct EZH2 contact, by the methyl-H3-K27 chromatin mark, and/or by other intermediary factors. The observation that wild-type EZH2, but not a mutant lacking the SET domain, could recruit DNMTs [50] implies that PRC2 catalytic function is involved. However, once a target gene becomes densely CpG hypermethylated, its maintained DNA methylation and silencing may no longer require EZH2 [51].

Subsequent studies expanded the EZH2-DNA methylation link by comparing chromatin states in cancer cells versus normal cells and by investigating many more target genes [52–54]. These gene profile comparisons reveal that EZH2 target genes, which display Me-H3-K27 in normal cells, are highly correlated with genes that become abnormally hypermethylated in cancer cells. Collectively, these studies suggest that EZH2 pre-marks certain genes to later become CpG hypermethylated during cellular transformation. Thus, genes that acquire Me-H3-K27 during normal development are somehow predisposed for DNA hypermethylation and conversion to "deep" silencing [7,52] in the presence of oncogenic cues. These cues presumably include abnormally high EZH2 levels but additional factors are likely involved. One group suggests that chromatin modifications on H3-K9 may also contribute during this transition [52]. Although much of this data relies on genes hypermethylated in colon cancer, similar findings were reported for other tumor types including prostate, liver, lung, ovarian and breast [53,54]. A recent study has also described functional connections between EZH2 and DNA methylation in acute promyelocytic leukemia [55].

4.2. EZH2 links to histone deacetylation

Physical and functional links between EZH2 and histone deacetylases (HDACs; [56,57]) predate the basic discovery that PRC2 has histone methyltransferase activity. In human cells, PRC2 can physically associate with HDACs 1 and 2 [14,57] and PRC2-mediated transcriptional silencing is impeded by the HDAC inhibitor TSA [34,57]. The sum of the biochemical data suggests that HDACs are not core subunits of PRC2 [12–16] but transient interactions likely still provide functional synergy between these silencing enzymes in vivo. The precise mechanisms of this synergy at target gene chromatin are not yet clear. As illustrated in Fig. 2, HDACs could deacetylate H3-K27 to make the ε-amino group available for methylation by PRC2. Alternatively, HDACs could deacetylate other histone lysines, such as H3-K9, H3-K14 or H4-K8, to adjust the local histone code for silencing. Taken together, functional links between EZH2, HDACs, and DNMTs contribute to an emerging view that all three types of epigenetic silencing machinery contribute to abnormal control of gene expression in cancer cells.

5. Towards deciphering EZH2 mechanisms in cancer cells

Although there is a large body of data implicating EZH2 in cancers of many types (Table 1), relatively little is known about molecular mechanisms of altered EZH2 function in cancer cells. It is important to fully understand: (1) biochemical changes that affect PRC2 composition and/or activity in cancer cells, (2) how chromatin states and expression of EZH2 target genes are altered in cancer cells, and eventually (3) how this altered expression profile contributes to oncogenesis. Recent progress in these areas, as well as possible cytoplasmic EZH2 function, is discussed below. Since the main mechanistic question concerns the consequences of EZH2 over-expression, it is worth emphasizing that there is normally little EZH2 in adult differentiated tissues [34,35,42]. In contrast to widespread EZH2 roles in early mouse development [58,59], post-embryonic EZH2 expression is limited [60,61]. Even when detected in adult tissues, EZH2 is concentrated in undifferentiated progenitor cell populations, such as hematopoietic cells of the pro-B lymphocyte lineage [62]. Thus, EZH2 "over-expression" in cancer tissues may reflect inappropriate EZH2 accumulation in cell types that normally lack it as opposed to merely adjusting EZH2 levels upwards in cells where it normally functions.

5.1. Altered forms of PRC2 in cancer cells

Different forms of the EZH2 complex occur in human cells, which are distinguished by the particular EED subunit included [63]. Specifically, there are four EED isoforms, distinguished by N-terminal extensions of differing lengths, that are produced from alternative translation start sites [63]. Canonical PRC2 complexes contain the longest EED isoform, called EED1, whereas PRC3 and PRC4 contain the shorter EED isoforms. Significantly, the PRC4 variant of the histone methyltransferase complex is selectively enriched in cancer cells versus normal cells and PRC4 assembly is favored when EZH2 is over-expressed in a cell line [45]. PRC4 differs biochemically from the other EZH2 complexes in several ways: (1) it contains the second largest EED isoform, EED2, (2) it also contains the NAD-dependent histone deacetylase, SirT1, and (3) it prefers to methylate histone H1-K26 rather than H3-K27. Since SirT1 can deacetylate H1-K26, its role in PRC4 may be to prepare this H1 residue for methylation [45]. This study raises the key prospect that PRC2 is compositionally and functionally reconfigured in a cancer-specific context. Furthermore, the results suggest that H1 methylation, rather than H3, might be a critical EZH2-sponsored modification in cancer cells. However, little is yet known about in vivo functions of H1-K26 methylation and an independent study using recombinant EZH2 complexes failed to detect H1 methylation when EED isoforms were varied [64]. Additional studies on a

wide array of tumor samples will be needed to assess if H1-K26 methylation and/or EED2 over-abundance are commonly observed during cancer progression. Intriguingly, a recent study finds that tri-methyl-H3-K27 levels are *decreased* in breast, ovarian and pancreatic cancer samples [65], which could reflect a shift in lysine substrate preference. The idea that cancer cells might preferentially deploy an altered form of PRC2 is clinically important because cancer-specific subunits or interactions could provide targets for specifically inhibiting aberrant PRC2 functions without adversely affecting normal roles.

5.2. Altered expression of EZH2 target genes: obligate silencing?

The predominant current view is that EZH2 functions in cancer as a dedicated transcriptional repressor that hypersilences an array of target genes, including tumor suppressor genes. This view is supported by the cohort of PRC2-repressed genes linked to poor outcome in prostate cancer patients [39] and the coincidence of EZH2 target genes and genes hypersilenced by DNA methylation in cancer, as discussed above [52–54]. Moreover, genome-wide studies revealed that only a very small percentage (<1% in F9 teratocarcinoma cells) of genes are simultaneously bound by PRC2 and RNA polymerase II [66,67], which also implies nearly universal PRC2 silencing. However, studies of EZH2 in other contexts have yielded occasional evidence for roles in target gene activation. Although the majority of responding genes were up-regulated after PRC2 knockdown in colon cancer cells, a substantial minority were down-regulated, including some direct PRC2 targets [46]. In human fibroblasts, PRC2 subunits were required for expression rather than silencing of proliferation genes [35] and a role for PRC2 in activating certain target genes in mouse ES cells has been considered [68]. Moreover, a recent report describes EZH2 as an activator of cell cycle control genes in breast cancer cells [69] and this activator function appears independent of the SET domain.

These varying results emphasize that basic transcriptional mechanisms of EZH2 in cancer remain an open question. Although most evidence favors a predominant EZH2 role in silencing, further work is needed to address alternatives. Indeed, a potential dual role for the *Drosophila* version of EZH2 in silencing and activation has long been considered [70,71].

A related issue concerns just what happens when excess EZH2 accumulates in a cancer cell. The simplest view might be that it assembles to create abnormally high levels of PRC2, which then hypermethylates H3-K27 and hypersilences target genes. This outcome assumes that SUZ12 and EED are present in sufficient quantities to partner with the extra EZH2. Alternatively, an imbalance of PRC2 subunits could lead to accumulation of unassembled EZH2 or trigger production of aberrant PRC2 subcomplexes. Since EZH2 enzyme function requires assembly with its partners, free EZH2 or catalytically inactive subcomplexes could act as dominant-negatives that desilence rather than hypersilence target genes. Thus, like the PRC2 isoforms described above [45], it is important to address if and how shifts in PRC2 subunit stoichiometries might impact target gene responses in cancer cells.

5.3. EZH2 recruitment to target genes

Since none of the PRC2 subunits are sequence-specific DNA-binding proteins, it is not known how EZH2 histone methyltransferase is recruited to target genes. In *Drosophila*, Polycomb response elements (PREs) have been delimited using reporter assays and several DNA-binding proteins are implicated in recruiting PRC2 (reviewed in [72]). However, mammalian PREs have yet to be precisely defined. A good candidate for a mammalian PRC2-targeting factor is YY1, whose *Drosophila* homolog, pleiohomeotic (PHO), is the best-characterized recruiter of PRC2 in flies [73]. Indeed, YY1 is needed for H3-K27 methylation of target genes in muscle cells [74]. However, this role may be cell-type specific as there is little overlap between YY1 targets and PRC2 targets in mouse ES cells [66]. Instead, Oct4 has been implicated in PRC2 targeting in ES cells ([66,75]; see below). Another study, using promyelocytic leukemia cells, shows that PRC2 can be recruited to target loci through interaction with the PML-RARα fusion protein but not with wild-type RARα [55]. Finally, a long non-coding RNA has been implicated in targeting PRC2 to the human *HoxD* cluster [76]. More work is needed to define sequence elements and mechanisms that recruit PRC2 to target loci in mammals. Taken together, the current data suggest that recruitment factors are likely to vary in different cell types and contexts.

5.4. EZH2 silencing and partnership with PRC1

Numerous studies have established that PRC2 histone methyltransferase frequently partners with another Polycomb complex, called PRC1, to achieve silencing in many systems ([8,77] for reviews). This partnership in human cells is supported by genome-wide mapping, which reveals frequent co-occupancy of target genes by both PRC2 and PRC1 [78,79]. The core subunits of human PRC1 are Polycomb (PC), polyhomeotic (PH), the oncoprotein BMI-1, and RING1, with the precise composition varying due to alternative subunit family members [80]. The chromodomain of the PC subunit can bind to tri-methyl-H3-K27 [81], which has inspired models wherein PRC1 is recruited to target chromatin by affinity for the methyl mark deposited by PRC2 [12]. In support of this idea, studies in both human cells and *Drosophila* have found that PRC2 function is needed for PRC1 recruitment to target genes [15,78]. Other models suggest that PRC1 interaction with K27-methylated nucleosomes is primarily to form intralocus chromatin loops that contribute to further histone modifications and silencing [72,77]. Despite these variations, it is worth emphasizing that PRC1 is commonly viewed as the direct executor of Polycomb silencing at many target genes. The actual Polycomb silencing mechanism(s), which may include blocks to nucleosome remodelling [80], chromatin compaction [82], histone H2A ubiquitylation [83] and/or blocks to transcription elongation [84–86] remain to be fully elucidated. More work is also needed to determine if the output of abnormal EZH2 function in cancer cells depends upon or is independent of PRC1 partnership. As illustrated in Fig. 2, (see also [7]), a PRC1-independent mechanism could feature PRC2-mediated histone methylation leading to permanent silencing by CpG DNA methylation.

5.5. EZH2 function at the actin cytoskeleton

Although the vast majority of work on EZH2 is focused on chromatin regulation, EZH2, SU(Z)12 and EED are also detected in the cytoplasm of mouse and human cells and the methyltransferase is implicated in controlling actin polymerization in response to cell signalling [87]. Correspondingly, EZH2 overabundance could affect cytoskeletal-based behaviors such as migration and invasion of cancer cells. Indeed, recent reports indicate that EZH2 over-accumulation in prostate cancer cell nuclei is paralleled by cytoplasmic overabundance and knockdown suggests that EZH2 influences invasiveness and F-actin polymerization in these cells [88,89]. Thus, nuclear and cytoplasmic functions could both contribute to EZH2-mediated alterations in cancer cells.

6. EZH2 functions in stem cell biology: connections to cancer

6.1. Differentiation gene silencing in stem cell maintenance

Genome-wide searches for PRC2 target genes have been performed by chromatin immunoprecipitation of EZH2 or SUZ12 coupled to genomic microarray hybridizations (ChIP-on-Chip). The first of these ChIP-on-Chip studies identified PRC2 target genes in colon cancer cells [46] and subsequent genome-wide searches have been conducted in human embryo fibroblasts [79], breast cancer cells [66], and both mouse and human embryonic stem cells [66,67,78]. A fundamental finding from these studies is that PRC2 target genes are highly enriched for transcription factors and signalling components that control cell differentiation. This preferential PRC2 role in developmental networks is also evident from genome-wide searches in *Drosophila* [90–92]. Thus, the originally defined function of fly Polycomb proteins as silencers of Hox differentiation factors [93] has been expanded to include dozens of other differentiation factor targets such as members of the Gata, Sox, Fox, Pou and Pax transcription factor families and components in Wnt, TGF-β, Notch, FGF and retinoic acid signalling [67,78,79]. Although these myriad factors function in many different tissues including neuronal, bone, muscle, blood and skin, a common role is in converting stem cell-like progenitors into more differentiated cell types within these and other lineages.

The analysis of PRC2 distribution and function in embryonic stem (ES) cells is particularly striking and informative [67,78]. Three transcription factors, Oct4, Sox2 and Nanog, play critical roles in programming ES cell gene expression to maintain pluripotency [94]. In general, these factors promote expression of proliferation genes and they silence differentiation genes. The very high correspondence between silenced genes bound by these three factors with those that also bind PRC2 [67,78] suggests that PRC2 is a key corepressor in ES cells. Indeed, loss of PRC2 derepresses ES cell differentiation genes [67,78] and knockdown of Oct4 disrupts PRC2 association with target genes [66,75]. Thus, PRC2 is implicated in ES cell self-renewal as an inhibitor of the differentiation program. This role could explain why ES cell lines cannot be derived from null EZH2 mouse embryos [59]. A similar PRC2 role is envisioned in progenitor cell types such as multipotential neuronal or hematopoietic cells; although the target genes vary in different lineages, the common function would be stem cell maintenance via PRC2 silencing of differentiation genes. Recent studies show that differentiation gene silencing in mouse ES cells also requires the PRC1 subunit, RING1 [75,95], which suggests that stem cell maintenance requires PRC2/PRC1 collaboration as seen in other examples of PcG silencing [8,77].

Intriguingly, the PRC2 reaction product, methyl-H3-K27, is part of a specialized chromatin state, termed a "bivalent domain," that marks the silenced ES cell differentiation genes [96]. This state features nucleosomes methylated on histone H3-K4 encompassed by larger expanses of chromatin bearing methylated H3-K27. Their simultaneous accumulation is unusual since methylated H3-K4 generally promotes activation whereas methylated H3-K27 leads to silencing. In this case, methyl-H3-K27 appears to "win out" since target genes in this state remain off. These bivalent domains could provide a sensitized state whereby crucial differentiation genes are kept silent but nevertheless poised for rapid activation in response to differentiation stimuli. For example, erasure of H3-K27 methylation by lysine-specific demethylases [97,98] could resolve bivalent domains to trigger gene activation.

6.2. EZH2, stem cell properties, and cancer

The many parallels between stem cells and tumor cells, including high proliferation rates and differentiation capacity, have prompted hypotheses that undifferentiated or dedifferentiated precursor cells may play key roles in oncogenesis. Growing evidence in favor of this "stem cell origin of cancer" hypothesis has been extensively reviewed [99–102]. In this context, the role of EZH2 in promoting self-renewal and impeding differentiation of ES cells suggests potentially similar roles during cancer progression. In molecular terms, tumor suppressor genes may resemble stem cell differentiation genes by featuring flexible chromatin states that are initially "transcription-ready" [52]. During oncogenesis, this plastic state could progress to permanent silencing, for example by further acquisition of DNA methylation [52–54]. The trigger for this proposed chromatin transition is not known but presumably involves EZH2 over-expression. Since there is normally little EZH2 in differentiated adult tissues, EZH2 overabundance could shift expression profiles to promote a return to or reinforcement of a stem cell-like state. Remarkably, a direct link between poorly differentiated human tumors and the ES cell state is provided by a shared gene expression signature defined in part by PRC2 target genes and Oct4/Sox2/Nanog target genes [103]. Clearly, the pace of discovery at the intersection between stem cell biology, chromatin, and cancer epigenetics is accelerating. These rapidly expanding topics are nicely integrated in these recent review articles [7,11].

7. Towards epigenetic therapy including EZH2 blockade

The epigenetic silencing of tumor suppressor genes in cancer has inspired potential therapeutic strategies that use inhibitors of epigenetic enzymes ([4,104,105] for reviews). A goal of epigenetic therapy is to achieve pharmacological reactivation of abnormally silenced genes in cancer patients, which could arrest or even reverse processes contributing to tumorigenesis. There are many inhibitors available that target either DNMTs or HDACs and clinical trials are underway to assess these [104–108]. Since epigenetic enzymes often synergize in vivo, as discussed above, there is also great interest in testing combined inhibitor treatments that target more than one epigenetic enzyme. In cell and animal models, simultaneous disruption of DNMTs and HDACs has produced encouraging results on gene reactivation (reviewed in [4]). One of the principles to emerge is that DNA methylation appears to dominate silencing, such that sequential treatment with DNMT inhibitor followed by HDAC inhibitor is preferred for optimal gene reactivation [109,110]. Some early clinical trials are beginning to test efficacies of combined DNMT/HDAC inhibitors in leukemia patients [107], with at least one study reporting reversal of DNA methylation and hematological improvement [111].

Similar to DNMTs and HDACs, EZH2 histone methyltransferase has emerged as a key target in potential epigenetic strategies. However, specific inhibitors of EZH2 histone methyltransferase have not yet been described. Although small molecule inhibitors of other histone methyltransferases are emerging [112], the most encouraging inhibitory agent of PRC2 reported so far is deazaneplanocin A (DZNep), which works through an indirect mechanism [113]. DZNep is an *S*-adenosylhomocysteine (Ado-Hcy) hydrolase inhibitor; it causes Ado-Hcy levels to rise, which blocks *S*-adenosylmethinone-dependent methyltransferases through by-product inhibition. Importantly, DZNep can deplete PRC2 subunits in breast cancer cell lines and reactivate PRC2-silenced genes [113]. However, since this type of inhibitor may affect many processes that require methyl transfer, there are concerns about its specificity as a potential therapeutic. Alternative strategies for designing specific

PRC2 inhibitors would include targeting the EZH2 active site and/or surfaces for key subunit interactions. A recent report provides structural data on the interface that mediates EZH2–EED interaction in PRC2 [114]. A high-resolution structure for the EZH2 SET domain, which houses the methyltransferase active site, would profoundly influence design of small molecule inhibitors specific for PRC2. The availability of these histone methyltransferase inhibitors should expand the repertoire of new possibilities in combined epigenetic therapy.

Acknowledgments

We thank members of our laboratories for discussions and critical comments on the manuscript. Work in our laboratories is supported by grants from the National Institutes of Health and Department of Defense to J.S. and from the National Institutes of Health and American Cancer Society to C.L.

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Cyclin-dependent kinases regulate epigenetic gene silencing through phosphorylation of EZH2

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The Polycomb group (PcG) protein, enhancer of zeste homologue 2 (EZH2), has an essential role in promoting histone H3 lysine 27 trimethylation (H3K27me3) and epigenetic gene silencing[1–4]. This function of EZH2 is important for cell proliferation and inhibition of cell differentiation, and is implicated in cancer progression[5–10]. Here, we demonstrate that under physiological conditions, cyclin-dependent kinase 1 (CDK1) and cyclin-dependent kinase 2 (CDK2) phosphorylate EZH2 at Thr 350 in an evolutionarily conserved motif. Phosphorylation of Thr 350 is important for recruitment of EZH2 and maintenance of H3K27me3 levels at EZH2-target loci. Blockage of Thr 350 phosphorylation not only diminishes the global effect of EZH2 on gene silencing, it also mitigates EZH2-mediated cell proliferation and migration. These results demonstrate that CDK-mediated phosphorylation is a key mechanism governing EZH2 function and that there is a link between the cell-cycle machinery and epigenetic gene silencing.

PcG proteins are important regulators of epigenetic gene silencing[8–10] and have key roles in developmental patterning, X-chromosome inactivation and stem cell maintenance[5, 6, 11]. Many of the proteins in this family function in two distinct protein complexes termed Polycomb-repressive complex 1 (PRC1) and Polycomb-repressive complex 2 (PRC2). PRC2 contains four core subunits of EZH2, EED, SUZ12 and RbAp 48(46) in humans or E(z), esc, Su(z)12 and Nurf55 in flies[1–4]. EZH2 is the catalytic subunit of PRC2 and contains a SET domain responsible for H3K27me3[1–4]. This chromatin mark is commonly associated with silencing of differentiation genes in organisms ranging from plants and flies to humans[8–10], suggesting that EZH2 is a master suppressor of cell differentiation.

Many studies also link EZH2 to oncogenesis[7, 12]. Compared with corresponding normal tissues, EZH2 levels are frequently elevated in numerous human cancers, including prostate cancer[7]. The abundance of EZH2 correlates with advanced tumour stage and poor prognosis for the patient[7] and forced expression of EZH2 promotes cancer cell proliferation and migration. Conversely, knockdown of EZH2 by RNA interference inhibits cancer cell proliferation and migration[7, 13]. The role of EZH2 in tumorigenesis may reflect its activity in silencing of tumour suppressor genes, such as *p16*^{INK4A}, *ADRB2* and *DAB2IP*[14–16].

Few studies have been performed to understand how the function of this regulatory protein is itself controlled. *EZH2* gene transcription is negatively regulated by the tumour suppressor protein, RB, and the microRNA, *miR-101* (refs 13 and 17). Akt phosphorylates EZH2 at Ser 21 and inhibits its methyltransferase activity[18]. However, it is unclear how the function of EZH2 is positively regulated, and maintained, in proliferative cells.

EZH2 expression and activity are higher in proliferating, rather than fully differentiated, cells and tissues[17,19,20]. Accordingly, EZH2 has a crucial role in the maintenance of stem cell pluripotency and suppression of cell differentiation[6,11,21]. As EZH2 commonly functions in highly proliferative cells that have high CDK activities, we hypothesized that EZH2 might functionally interact with CDKs in proliferative cells. Indeed, EZH2 harbours one perfectly matched (Thr 350) and two imperfectly matched (Thr 421 and Thr 492) CDK phosphorylation motifs (K(R)S(T)PXK(R), where X is any residue[22]; Supplementary Information, Fig. S1a). To assess phosphorylation by CDKs, GST fusions of the amino terminus (amino-acid residues 1–559) and carboxy terminus (amino-acid residues 560–741) of EZH2 were used in *in vitro* protein-kinase assays. The EZH2 N-terminal fragment was phosphorylated by the CDK1–cyclin B1 complex, but the C-terminal fragment was not (Fig. 1a). As expected, histone H1B, a known CDK1 substrate, was readily phosphorylated in these assays, whereas no phosphorylation of the control glutathione S-transferase (GST) protein was observed (Fig. 1a). Mutation of Thr 350 to alanine (T350A) resulted in approximately 60% reduction in phosphorylation of the N-terminal EZH2 fragment mediated by CDK1 (Fig. 1b). In contrast, approximately 30% or no reduction in phosphorylation was observed when T421A and T492A mutants were used as substrates (Fig. 1b). This suggests that Thr 350 in EZH2 is the major site phosphorylated

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Received 08 July 2010; accepted 06 September 2010; published online 10 October 2010; DOI: 10.1038/ncb2116

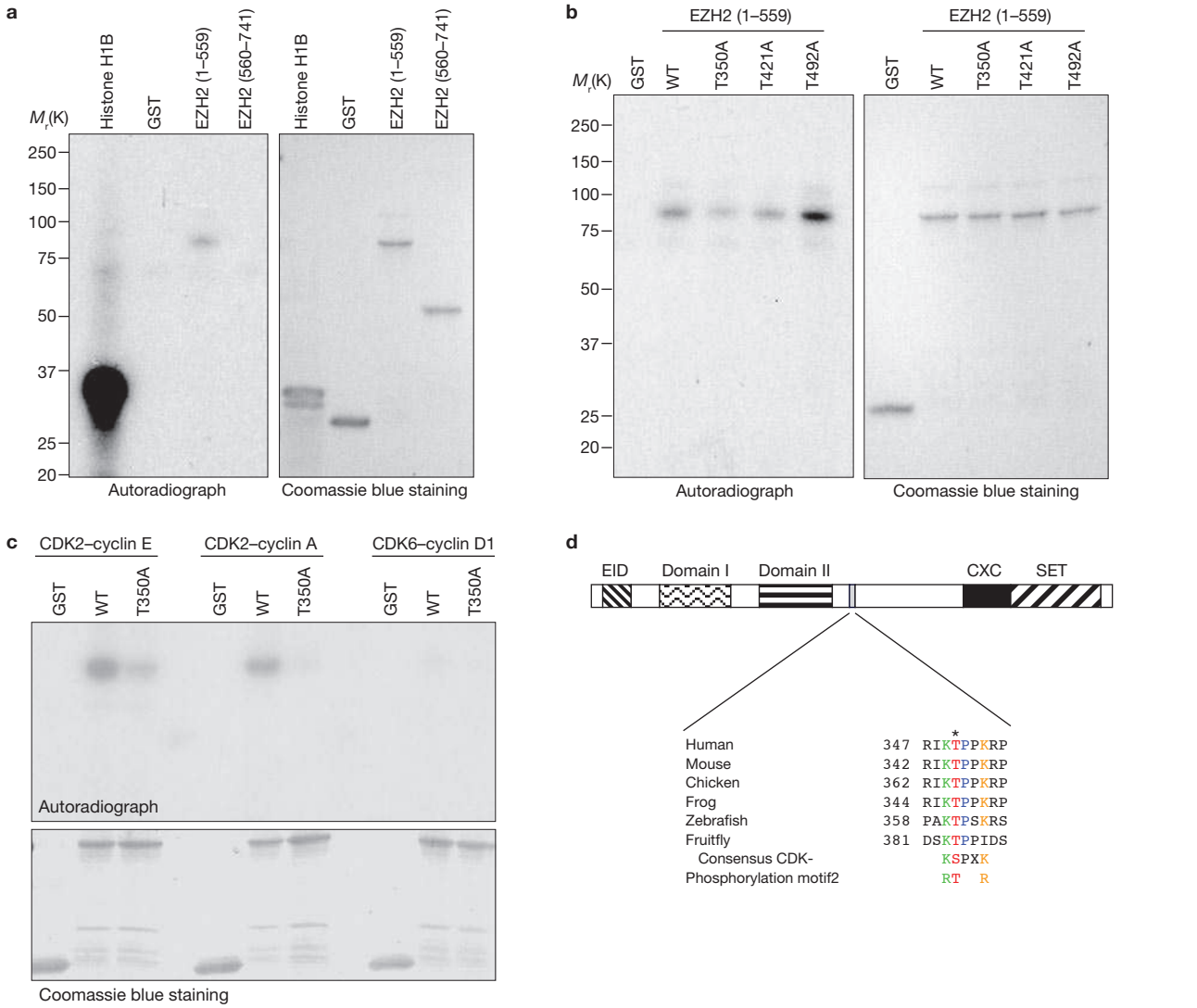

Figure 1 CDK1 and CDK2 phosphorylate EZH2 at Thr 350 *in vitro*. (**a**) Left: *in vitro* kinase assay. Recombinant CDK1–cyclin B1 protein complex was incubated with [γ-^{32}P]ATP and the indicated substrates. Reaction samples were resolved by SDS–PAGE and autoradiography. Right: protein substrates indicated by Coomassie blue staining. (**b**) Left: *in vitro* CDK1 kinase assay using a wild-type (WT) EZH2 GST-fusion protein fragment (amino-acid residues 1–559), and T350A, T421A, and T492A mutants of EZH2 (amino-acid residues 1–559), as substrates. Right: protein substrates indicated by Coomassie blue staining. (**c**) Top: *in vitro* CDK2 and CDK6 kinase assays using GST, a wild-type EZH2 GST-fusion protein segment (residues 1–559) and a EZH2 (residues 1–599) T350A mutant, as substrates. Bottom: protein substrates indicated by Coomassie blue staining. (**d**) Comparison of the amino-acid sequence of EZH2 homologues near the CDK phosphorylation site (Thr 350 indicated by asterisk). Uncropped images of blot are shown in Supplementary Information, Fig. S6a.

by the CDK1–cyclin B1 complex *in vitro*. Further analysis showed that CDK2–cyclin E and CDK2–cyclin A, but not CDK6–cyclin D1, can also phosphorylate EZH2, and that this phosphorylation is largely or completely abolished by the T350A mutation (Fig. 1c). These data indicate that the EZH2 protein can be specifically phosphorylated at the Thr 350 residue by different CDKs *in vitro*. Notably, this residue is present in a consensus CDK phosphorylation motif that is evolutionarily conserved from fruit flies to humans (Fig. 1d; although the putative CDK site in the fruitfly homologue of EZH2 is imperfectly matched with the CDK consensus motif there is a similar motif in the mitosis regulatory protein, nucleophosmin (NPM or B23) that has been shown to be phosphorylated by CDK1; ref. 30).

To determine whether CDK1 and CDK2 can phosphorylate EZH2 at Thr 350 *in vivo*, an antibody specific to phosphorylated Thr 350 (anti-Thr 350–P) was raised and purified. The antibody reacted with wild-type but not EZH2^{T350A} in both 293T (Fig. 2a) and prostate cancer LNCaP cells (Supplementary Information, Fig. S1b). This reaction was blocked by a peptide containing the phosphorylated Thr 350, but not by the corresponding nonphosphorylated peptide (Supplementary Information, Fig. S1c). Treatment of cellular proteins with λ protein phosphatase completely abolished the reaction of this antibody with EZH2 (Fig. 2b), confirming that the anti-Thr 350–P antibody is specific to phosphorylated Thr 350. Ectopic expression of CDK1–cyclin B1 or CDK2–cyclin E substantially increased Thr 350 phosphorylation of both endogenous and exogenous wild-type EZH2, but not EZH2^{T350A}, in LNCaP cells (Fig. 2c and Supplementary Information, Fig. S1b). Thr 350 phosphorylation of EZH2 was inhibited in cells overexpressing the CDK inhibitors, p21^{WAF1} (Fig. 2c) and p27^{KIP1} (Supplementary Information, Fig. S1b). Thr 350 phosphorylation of endogenous EZH2 was substantially reduced by knockdown of endogenous CDK1 and

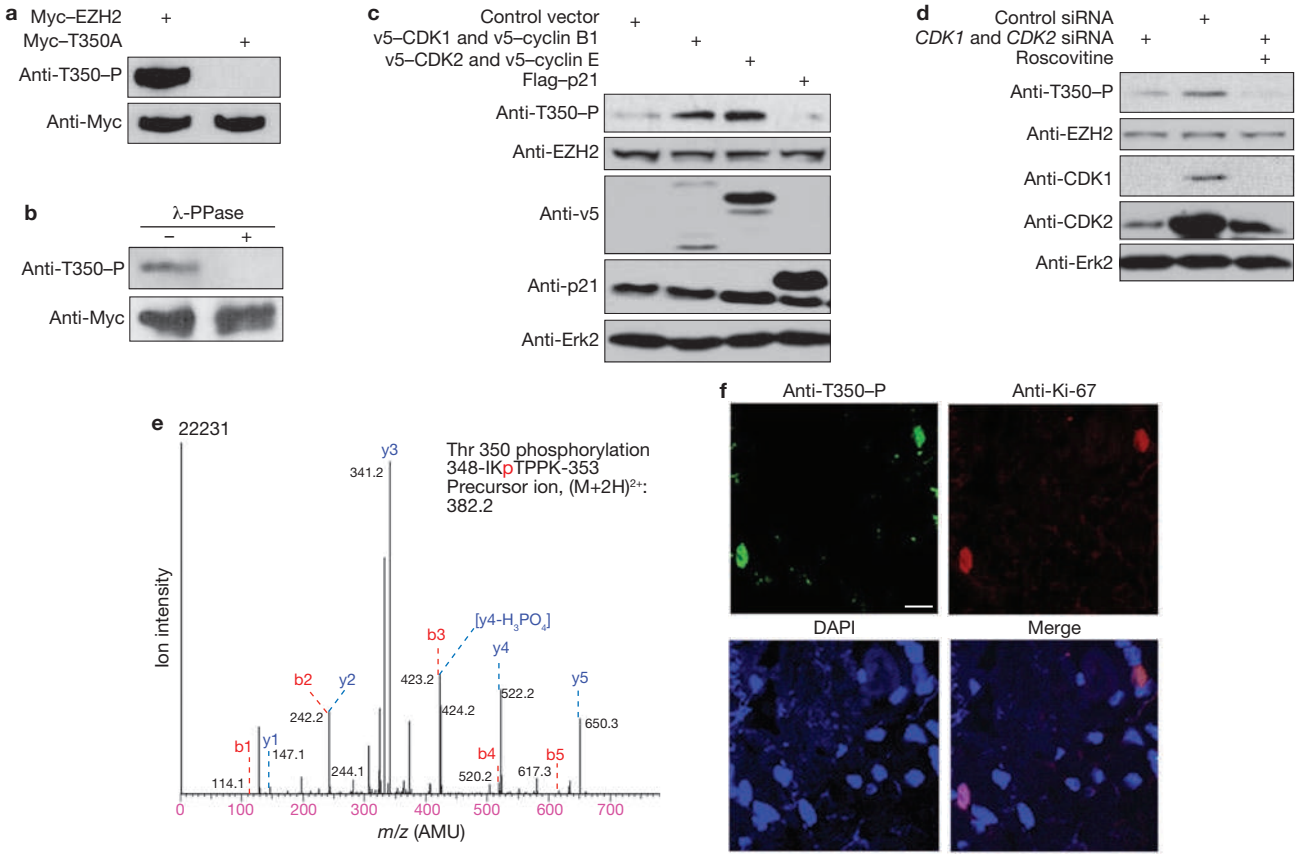

Figure 2 CDK1 and CDK2 phosphorylate EZH2 at Thr 350 *in vivo*. (**a**) 293T cells were transfected with plasmids to express Myc-tagged wild-type EZH2 or a EZH2^{T350A} mutant. Ectopically expressed EZH2 proteins were immunoprecipitated with anti-Myc, and resolved by western blot using antibody raised against phosphorylated Thr 350 (anti-T350–P) or anti-Myc. (**b**) EZH2 was immunoprecipitated from 293T cells expressing Myc–EZH2. Immunoprecipitated EZH2 proteins were subjected to λ protein phosphatase treatment and resolved by western-blot analysis with anti-T350–P or anti-Myc antibodies. (**c**) LNCaP cells were transfected with plasmids expressing v5–CDK1 and v5–cyclin B1, v5–CDK2 and v5–cyclin E, or Flag–p21^{WAF1}. Thr 350 phosphorylation of endogenous EZH2 was detected by the anti-T350–P antibody. Immunoblotting of extracellular signal-regulated kinase 2 (Erk2) was included as a loading control. (**d**) LNCaP cells were transfected with siRNAs against *CDK1* and *CDK2* for 48 h and then treated with or without the CDK inhibitor roscovitine, as indicated. Endogenous EZH2 Thr 350 phosphorylation was detected by the anti-T350–P antibody. (**e**) Lysates from 293T cells were immunoprecipitated with the anti-T350–P antibody and resolved by SDS–PAGE gel analysis. The EZH2 band was excised and analysed by LC–MS/MS mass spectrometry. The MS/MS spectrum of the double-charged ion (*m/z* 382.2) shows that the Thr 350 residue is phosphorylated (low case p in red) in the peptide 348-IKTPPK-353. The b ions (b1–b5) are the fragmentation ions containing the N terminus of the peptide, whereas the y ions (y1–y5) are the fragmentation ions containing the C terminus of the peptide. (**f**) Representative immunofluorescence microscopy images of primary human prostate tumours using anti-T350–P, anti-Ki-67, and DAPI to visualize nuclei ($n = 12$). Scale bar, 10 μm. Uncropped images of blots are shown in Supplementary Information, Fig. S6a.

CDK2, and this effect was enhanced by further treatment with the CDK inhibitor, roscovitine (Fig. 2d). Thr 350 phosphorylation of both endogenous and ectopically expressed EZH2 in 293T cells was confirmed by mass spectrometry analysis (Fig. 2e and Supplementary Information, Fig. S1d and S1e). Furthermore, Thr 350-phosphorylated EZH2 was invariably co-localized with the proliferation marker Ki-67 in human prostate tumours (Fig. 2f). We also found that CDK1 and CDK2 interact with EZH2 *in vitro* and *in vivo* (Supplementary Information, Fig. S2). These data indicate that CDKs can phosphorylate EZH2 at Thr 350 under various physiological and pathological conditions.

The biological function of EZH2 is primarily reflected by its global repression of gene transcription[7,11]. Thus, we performed microarray analysis to gain molecular insights into the effect of EZH2 Thr 350 phosphorylation on gene expression in mammalian cells. Endogenous EZH2 was knocked down by an *EZH2*-specific siRNA (*EZ4* siRNA), or restored to physiological levels by ectopically expressing siRNA-resistant wild-type EZH2 (EZH2^{SR}) or a siRNA-resistant EZH2^{T350A} mutant (T350A^{SR}) in LNCaP cells (Supplementary Information, Fig. S3a and S3b). mRNA samples were then collected for oligonucleotide microarray profiling analysis. For comparison, microarray analysis was performed in LNCaP cells treated with the CDK inhibitor, roscovitine. Additionally, it has been shown previously that histone deacetylase (HDAC) proteins can physically interact with the PRC2 complex[23], and treatment of cells with the HDAC inhibitor trichostatin A (TSA) blocks EZH2-mediated gene silencing[7,23]. Therefore, as a positive control, we also performed microarray analysis of LNCaP cells treated with TSA. As demonstrated in Figure 3a (lanes 2 and 3), a large set of genes were transcriptionally derepressed by EZH2 knockdown and repressed again in cells with the restored expression of wild-type EZH2. Consistent with the role of HDACs in concert with the PRC2 complex[7,23], inhibition of HDACs by TSA also

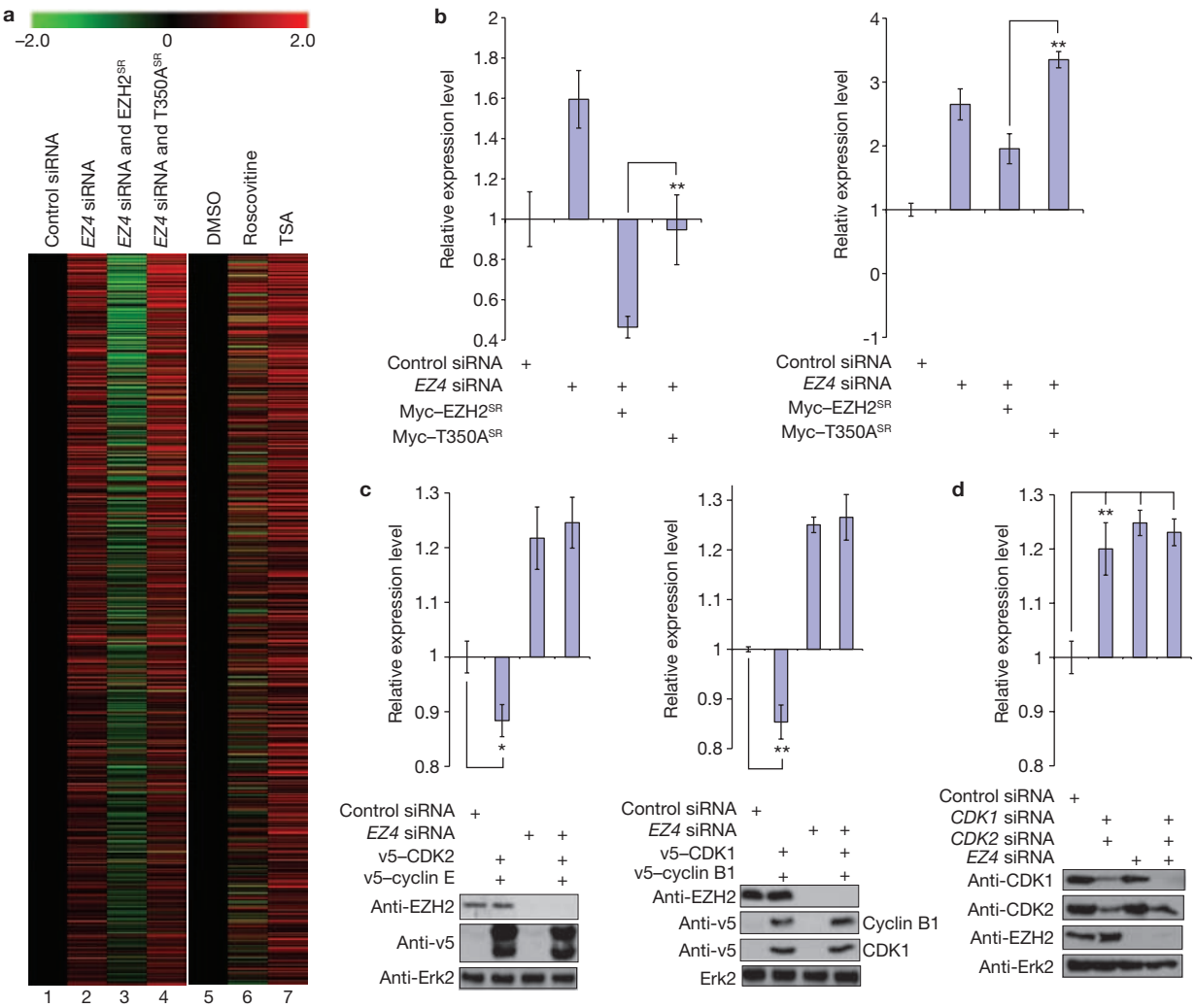

Figure 3 The effect of Thr 350 phosphorylation on EZH2-mediated repression of its target-genes. (**a**) Hierarchical clustering of 6,450 genes (represented by 10,276 probe-sets) that exhibited expression differences in LNCaP cells. Lanes 1–4; cells were transfected with *EZH2*-specific siRNA (*EZ4*) and vectors expressing siRNA-resistant EZH2 or a siRNA-resistant EZH2^{T350A} mutant, as indicated. Lanes 5–7; cells were treated with a CDK inhibitor or HDAC inhibitor. Gene profiling data from cells transfected with the indicated siRNA and vectors (lanes 2, 3 and 4) were normalized to that in cells transfected with control siRNA (lane 1), and the data from drug-treatment experiments (lanes 6 and 7) were normalized to vehicle (DMSO) treatment (lane 5). Red and green represent upregulation and downregulation, respectively, as indicated in the scale at the top. Wild-type EZH2 and EZH2^{T350A} mutant proteins were expressed at comparable levels (Supplementary Information, Fig. S3b). Experiments were performed in duplicate ($n = 2$). (**b**) LNCaP cells were transfected with *EZ4* siRNA or plasmids expressing Myc-tagged, siRNA-resistant EZH2 or Myc-tagged, siRNA-resistant EZH2^{T350A}, as indicated (empty vectors were used as a control). At 60 h after transfection, expression of *HOXA9* (left) and *DAB2IP* (right) were analysed by real-time RT–PCR. Asterisks indicate $P < 0.01$. Experiments were performed in triplicate ($n = 3$). (**c**) Prostate cancer cells were transfected with plasmids expressing v5–CDK2 and v5–cyclin E (DU145 cells, left), v5–CDK1 and v5–cyclin B1 (PC-3 cells, right), or an empty control vector, in combination with control or *EZH2* siRNA. At 72 h after transfection, expression of *HOXA9* was evaluated by real-time RT-PCR. Asterisk indicates $P < 0.05$, and double asterisks indicate $P < 0.01$. Experiments were done in triplicate ($n = 3$). Western blots (bottom) were used to identify expression of the indicated proteins. (**d**) DU145 cells were transfected with siRNAs against *CDK1*, *CDK2* and *EZH2* as indicated. At 72 h after transfection, expression of *HOXA9* was evaluated by real-time RT-PCR. Asterisks indicate $P < 0.01$. Experiments were carried out in triplicate ($n = 3$). Western blots (bottom) were used to identify expression of the indicated proteins. Uncropped images of blots are shown in Supplementary Information, Fig. S6a.

resulted in derepression of this set of EZH2 target-genes (Fig. 3a, lane 7). Most importantly, a great percentage (> 78%) of EZH2-target-genes failed to be repressed by expression of the siRNA-resistant EZH2^{T350A} mutant (Fig. 3a, lane 4). Similarly, we detected that more than 74% of EZH2-repressed genes are not repressed when EZH2^{T350A} is expressed in normal human BJ fibroblasts (Supplementary Information, Fig. S3c, d). Intriguingly, the majority (> 60%) of Thr 350 phosphorylation-regulated EZH2-target-genes were also affected by roscovitine treatment in LNCaP cells (Fig. 3a; lanes 4 and 6, and Supplementary Information, Fig. S3e), although, as expected, roscovitine treatment resulted in a much broader impact on gene expression (Supplementary Information, Fig. S3e). We conclude that CDK-induced Thr 350 phosphorylation of EZH2 is important for its genome-wide repression of gene transcription.

The *HOXA9* gene is a well-studied EZH2 repression target[1,18,24]. To determine whether EZH2 phosphorylation at Thr 350 affects *HOXA9* expression, endogenous EZH2 was knocked down or restored by ectopic

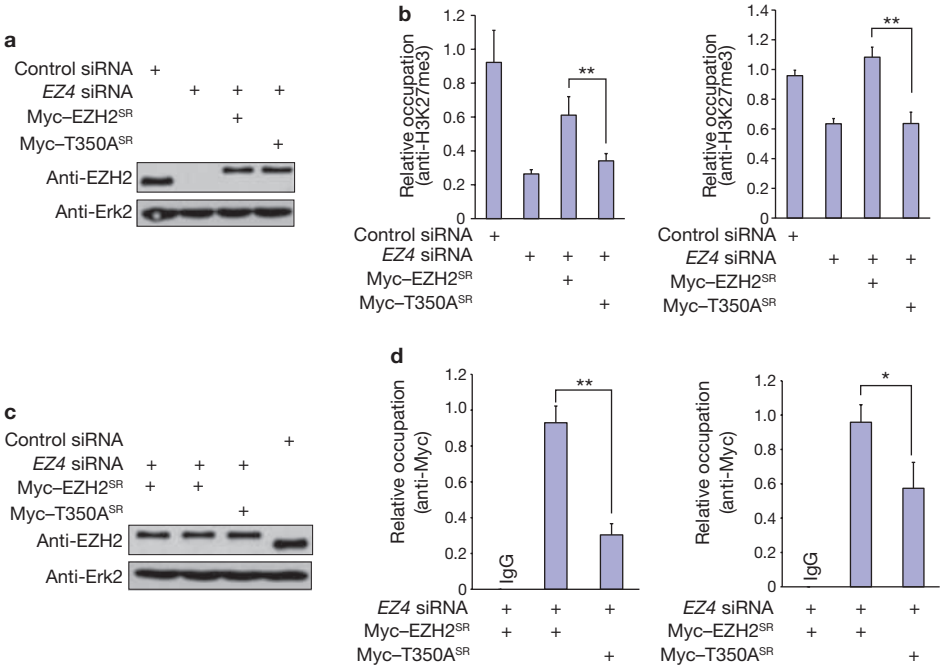

Figure 4 The effect of Thr 350 phosphorylation on H3K27me3 levels and EZH2 recruitment at EZH2-target-gene promoters. (**a**, **b**) LNCaP cells were transfected with control or *EZH2*-specific siRNA and plasmids expressing Myc-tagged, siRNA-resistant EZH2 or Myc-tagged, siRNA-resistant EZH2^{T350A} (or empty control plasmids), as indicated. (**a**) At 60 h after transfection, expression of EZH2 was examined by western blot. (**b**) H3K27me3 levels in promoters of the EZH2-target-genes, *HOXA9* (left) and *DAB2IP* (right), were assessed by ChIP assays using anti-H3K27me3 antibodies. Data are means ± s.d. from three individual experiments ($n = 3$). Asterisks indicate $P < 0.01$. (**c**, **d**) LNCaP cells were transfected with control or *EZH2*-specific siRNA and plasmids expressing Myc-tagged, siRNA-resistant EZH2 or Myc-tagged, siRNA-resistant EZH2^{T350A} (empty plasmids were used as a control), as indicated. (**c**) At 60 h after transfection, expression of endogenous and restored EZH2 was examined by western blot. (**d**) The binding of Myc–EZH2 and Myc–EZH2^{T350A} to *HOXA9* (left) and *DAB2IP* (right) promoters was examined by ChIP assays with anti-Myc antibody. Data are means ± s.d. from three individual experiments ($n = 3$). Asterisk indicates $P < 0.05$, double asterisks indicate $P < 0.01$. IgG; immunoglobulin G used as control antibody in ChIP assay. Uncropped images of blots are shown in Supplementary Information, Fig. S6b.

expression of siRNA-resistant wild-type EZH2 or EZH2^{T350A} using the strategy shown in Figure 3a and Supplementary Information, Figure S3b. As expected, knockdown of endogenous EZH2 resulted in an increase in *HOXA9* expression in LNCaP cells (Fig. 3b, left). *HOXA9* expression was repressed again by restored expression of wild-type EZH2. However, this effect was substantially compromised by the expression of EZH2^{T350A} (Fig. 3b, left). Overexpression of CDK2–cyclin E and CDK1–cyclin B1 also repressed *HOXA9* gene expression (Fig. 3c). This effect was abrogated by EZH2 knockdown (Fig. 3c). Moreover, silencing of endogenous CDK1 and CDK2 increased expression of *HOXA9* (Fig. 3d). No additive effect on *HOXA9* expression was observed in cells where CDK1, CDK2 and EZH2 were knocked down (Fig. 3d). Thus, these data suggest that CDK-mediated Thr 350 phosphorylation on EZH2 is important for its regulation of *HOXA9* expression.

Consistent with the fact that EZH2 is a strong promoter of cell proliferation and migration and a master repressor of cell differentiation[7,11,17,21,25,26], our microarray analysis revealed that many genes important for cell growth and differentiation are affected by EZH2 Thr 350 phosphorylation. Knockdown of EZH2 increased *DAB2IP* expression in LNCaP cells, consistent with previous reports that the putative tumour suppressor gene *DAB2IP* (which has a role in cancer cell proliferation and metastasis) is a EZH2 target[14,27] (Fig. 3b, right). This increase was diminished by restored expression of wild-type EZH2 but not the EZH2^{T350A} mutant (Fig. 3b, right). In addition to *HOXA9*, many other key developmental regulators, including transcription factors in the HOX, FOX and SOX families, are known targets of PRC2[11]. Our microarray data demonstrated that Thr 350 phosphorylation is important for EZH2-mediated repression of many of these genes (Supplementary Information, Fig. S3f and S3g). These data indicate that Thr 350 phosphorylation of EZH2 is important for its repression of genes either mediating differentiation or blocking cell proliferation and migration.

EZH2-promoted gene silencing is mediated primarily by its function in catalysing H3K27me3 in the promoters of its target-genes[1,18,24]. Consistently, chromatin immunoprecipitation (ChIP) analysis in LNCaP cells demonstrated that knockdown of EZH2 decreased the level of H3K27me3 in the promoters of *HOXA9* and *DAB2IP* (Fig. 4a, b). This effect was largely reversed by restored expression of wild-type EZH2, but not the EZH2^{T350A} mutant (Fig. 4a, b). Next, we assessed whether Thr 350 phosphorylation directly affects the enzymatic activity of EZH2. *In vitro* histone methyltransferase (HMTase) assays were performed using PRC2 complexes that were either immunoprecipitated from mammalian cells or reconstituted from proteins isolated after baculovirus-mediated expression in insect Sf9 cells. Surprisingly, no difference in HMTase activity was detected *in vitro* between wild-type EZH2 and the EZH2^{T350A} mutant (Supplementary Information, Fig. S4a and S4b). Furthermore, CDK-mediated phosphorylation of EZH2 did not alter core PRC2 complex formation in mammalian or insect cells (Supplementary Information, Fig. S4a and S4b), or the half-life of the EZH2 protein as assessed in LNCaP cells (Supplementary Information, Fig. S4c and S4d). Thus, the impact

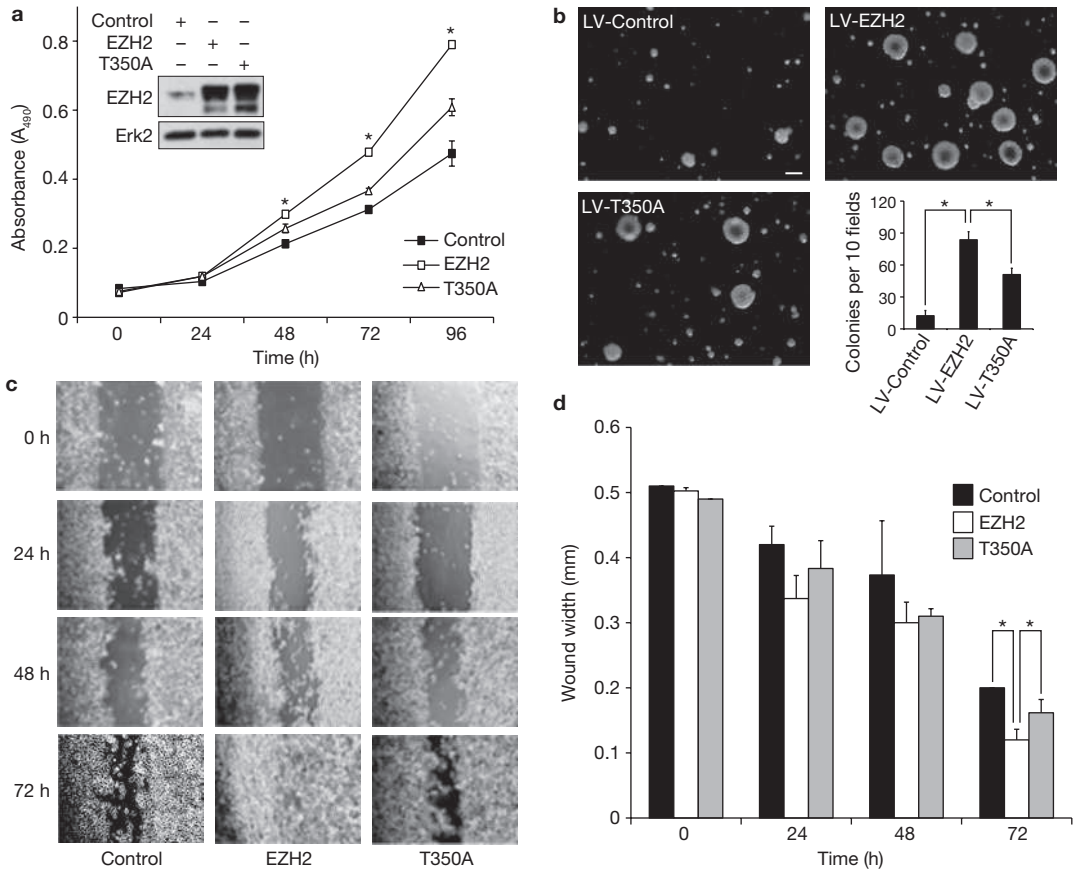

Figure 5 Phosphorylation of EZH2 Thr 350 is crucial for its function in promoting cell proliferation and migration. (**a**) RWPE-1 cells were transfected with empty control plasmids, or plasmids expressing wild-type EZH2 or the EZH2^{T350A} mutant. Graph shows cell proliferation, as monitored by MTS assay, at the indicated times after transfection. The levels of endogenous and ectopically expressed wild-type and EZH2^{T350A}-mutant proteins were detected with an anti-EZH2 antibody at 48 h after transfection by western blot (inset). Erk2 was included as a loading control. Asterisks indicate $P < 0.01$ when comparing cells transfected with plasmids expressing wild-type EZH2 to those transfected with plasmids expressing EZH2^{T350A}. Data are means ± s.d. from experiments with six replicates ($n = 6$) (**b**) Effects of EZH2 Thr 350 phosphorylation on anchorage-independent growth of 22Rv1 cells. Representative images of colonies formed by cells infected with lentiviral vectors expressing GFP (control), EZH2 or EZH2^{T350A}, and cultured in medium with agar for two weeks. Scale bar, 300 μm. Clones with the diameter larger than 300 μm in ten randomly selected fields were counted (graph, inset). Asterisk indicates $P < 0.01$. Data are means ± s.d. from three individual experiments ($n = 3$). (**c**, **d**) Cell migration evaluated by wound healing assay. RWPE-1 cells were transfected with empty control plasmids, or plasmids expressing EZH2 or EZH2^{T350A}. Artificial wounds were created on cells in confluence. Images were taken at 0, 24, 48 and 72 h after wound (**c**), and the wound widths were measured and quantified (**d**). Asterisk indicates $P < 0.01$. Data are means ± s.d. from five individual experiments ($n = 5$). Uncropped images of blot are shown in Supplementary Information, Fig. S6b.

of EZH2 Thr 350 phosphorylation on H3K27me3 levels in target-gene promoters (Fig. 4b) cannot be attributed to changes in stability, formation or intrinsic HMTase activity of PRC2. We performed ChIP assays to test for changes in PRC2 targeting. Indeed, the binding of EZH2^{T350A} to the promoters of *HOXA9* and *DAB2IP* was much lower, compared with wild-type EZH2 (Fig. 4c, d). These data suggest that EZH2 Thr 350 phosphorylation may affect PRC2 recruitment to its target loci in cells.

Previous studies demonstrated that EZH2 is frequently overexpressed in advanced human prostate cancers, and that ectopic expression of EZH2 promotes proliferation of immortalized RWPE-1 prostate epithelial cells and PC-3 prostate cancer cells[7], two cell-lines that express relatively low levels of endogenous EZH2 (Supplementary Information, Fig. S5a). Consistent with those studies, ectopic expression of wild-type EZH2 markedly augmented growth of RWPE-1 cells (Fig. 5a). However, EZH2-stimulated proliferation of RWPE-1 cells was largely attenuated by the T350A mutation (Fig. 5a). This attenuation was not because of differences between levels of the wild-type and mutated EZH2 proteins (Fig. 5a, inset). A similar result was obtained in PC-3 cells (Supplementary Information, Fig. S5b). Consistent with these observations, we demonstrated using soft-agar assay that ectopic expression of wild-type EZH2 markedly enhanced anchorage-independent growth of 22Rv1 prostate cancer cells (Fig. 5b). However, this effect was largely diminished in cells infected with lentiviruses expressing the EZH2^{T350A} mutant (Fig. 5b), although wild-type and mutated EZH2 proteins were expressed at comparable levels (Supplementary Information, Fig. S5c). In addition to cell proliferation, EZH2 also promotes cell migration[13,28]. Thus, we performed wound healing assays to determine whether Thr 350 phosphorylation affects the role of EZH2 in cell migration. Similarly to the previous report[13], expression of wild-type EZH2 significantly accelerated migration of RWPE-1 cells (Fig. 5c, d). However, the T350A mutation largely diminished EZH2-promoted migration in this cell line (Fig. 5c, d). Thus, Thr 350 phosphorylation contributes to the tumour-promoting functions of EZH2, including proliferation and migration.

Our data demonstrate that CDKs function as important positive regulators of EZH2 through phosphorylation at the Thr 350 residue. Notably, the motif containing Thr 350 is evolutionarily conserved, suggesting that this regulatory mechanism could be functional in other organisms. Although the T350A mutation does not alter the intrinsic HMTase activity of PRC2 as assessed by *in vitro* assays using HeLa polynucleosomes as a substrate, Thr 350 phosphorylation not only affects H3K27me3 levels in the EZH2 target loci examined, it also regulates the global effect of EZH2 on gene silencing in different cell types. Consistent with these observations, ablation of Thr 350 phosphorylation diminishes the binding of EZH2 to its target loci in cells. Thus, our data identify CDK1- and CDK2-mediated Thr 350 phosphorylation as an important mechanism in control of EZH2-mediated epigenetic gene silencing in mammalian cells.

The function of EZH2 is essential for silencing of differentiation factors, thereby making key contributions to maintenance of stem cell pluripotency[6,11,21]. We demonstrate that CDK phosphorylation is important for EZH2-mediated silencing of developmental regulators, such as members of the HOX, FOX and SOX families (Fig. 3 and Supplementary Information, Fig. S3f, g) that drive cell differentiation. Thus, CDK phosphorylation may augment the role of EZH2 in inhibiting these transcription factors and reinforce continued proliferation over differentiation. On cell cycle exit at certain stages of development, CDK stimulation of EZH2 would probably decline, which might facilitate desilencing of EZH2 targets and cell differentiation.

In addition to its role in repression of cell differentiation, EZH2 is also important for oncogenesis by regulating cancer cell proliferation and migration[7,15,17]. We provide evidence that Thr 350 phosphorylation is essential for these functions of EZH2 in prostate cancer cells. Because CDK activity is often elevated in human cancers[29], our data suggest that aberrant activation of CDKs may contribute to the aggressive phenotype of tumours by phosphorylating and maintaining the oncogenic and gene-silencing functions of EZH2. This regulatory node may serve as a viable therapeutic target to switch off the tumour-promoting functions of EZH2 in human cancers. □

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology/

Note: Supplementary Information is available on the Nature Cell Biology website

ACKNOWLEDGEMENTS

We would like to thank M. –C. Hung, Y. Zhang, H. Piwnica-Worms and L. Naldini for plasmids, R. A. Weinberg for BJ cells, K. Zhang for helping in the analysis of mass spectrometry data, and Z. Zhang for critical comments and suggestions. This work was supported in part by grants from the National Institutes of Health (CA134514 and CA130908 to H.H. and GM49850 to J.S.), the Department of Defense (W81XWH-07-1-0137 and W81XWH-09-1-622 to H.H. and W81XWH-07-1-0373 to J.S.), and a Brainstorm Award from University of Minnesota Masonic Cancer Center (to H.H.).

AUTHOR CONTRIBUTIONS

S.C. performed most of the experiments and analysis. L.R.B. generated mutation constructs. A.N.R. performed PRC2 complex purification and *in vitro* HMTase assays. Y.P. performed immunofluorescent chemistry. L.G. provided technical assistance. X.Z. and A.B. provided reagents and technical advices. H.H. conceived the study. S.C., J.A.S. and H.H. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Plasmids and reagents. Plasmids for Myc-tagged wild-type and SET domain-truncated (ΔSET) EZH2 were kindly provide by M. –C. Hung[18] (University of Texas, USA). EZH2^{T350A}, EZH2^{T350D}, EZH2^{T421A} and EZH2^{T492A} were generated by site-specific mutagenesis (Stratagene). Mammalian expression vectors of CDK1 and cyclin B1 were kindly provided by H. Piwnica-Worms[31] (Washington University School of Medicine, USA). v5-tagged expression vectors for the active mutants of CDK1 and CDK2, and cyclin B1 and cyclin E were described previously[22,32]. The N-terminal (amino-acid residues 1–559) and C-terminal (amino-acid residues 560–741) fragments of EZH2 were subcloned into pGEX-4T-1 vector (GE Healthcare) for production of GST–EZH2 fusion recombinant proteins. Amino-acid substitution mutants of the GST-tagged EZH2 N-terminal fragment were generated by PCR-based mutagenesis. Baculovirus expression vectors for mouse *Ezh2* (Enx-1) and human *EED*, *SUZ12* and *RbAp48* were generated by inserting full-length open reading frames of indicated cDNAs into pFastBac1 vector, as previously described[4]. Expression vector for p27^{KIP1} was described previously[33] and plasmid for Flag–tagged p21^{WAF1} was purchased from Addgene. The lentiviral expression vectors for GFP (green fluorescent protein; control), wild-type EZH2 and the EZH2^{T350A} mutant were constructed by subcloning corresponding cDNAs into the bidirectional vector as described[34,35]. Purified recombinant GST–CDK1, GST–CDK2, GST–cyclin B1, GST–cyclin E and histone H1B were obtained from Cell Signaling Technology.

Immunoprecipitation, western blotting and antibodies. Protein immunoprecipitations were carried out using an immunoprecipitation kit (Roche Applied Science) as previously described[22], and western blotting was performed as previously described[22]. A polyclonal antibody against the phosphorylated EZH2 at the Thr 350 residue was raised by immunizing rabbits with the phosphorylated human EZH2 peptide (AERIKpTPPKRPG), and purifying the antibody over a peptide-affinity column (Thermo scientific). Other antibodies are as follows: anti-CDK1, anti-cyclinB1, anti-EZH2, anti-histone H3 (Cell Signaling Technology); anti-CDK2, anti-p21^{WAF1}, anti-p27^{KIP1}, anti-Erk2 (Santa Cruz Biotechnology), anti-H3K27me3 (Millipore), anti-v5 (Invitrogen) and anti-Ki67 (Dako). Antibodies used for western blotting were diluted 1:1,000 and those used for immunofluorescent chemistry were diluted 1:500.

***In vitro* kinase assay.** Kinase assays were carried out in the presence of [γ-^{32}P] ATP by using an *in vitro* kinase buffer system from Cell Signaling Technology as previously described[22]. Briefly, recombinant CDK and cyclin complexes were incubated with various substrates at 30 °C for 45 min, and the reaction samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography. Photoshop software was used to quantify the intensity of each band.

Mass spectrometry, database searching and phosphorylation site localisation. A micro-capillary liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis of EZH2 protein samples was performed with an LTQ-Orbitrap mass spectrometer (ThermoFinnigan) by the Taplin Mass Spectrometry Facility at Harvard Medical School (https://gygi.med.harvard.edu/index.html) as previously described[36]. The product ion spectra generated by LC–MS/MS were searched against the National Center for Biotechnology Information (NCBI) databases for exact matches using the SEQUEST algorithm. The modification of 79.9663 mass units to serine, threonine and tyrosine was included in the database searches to determine phosphopeptides. Each phosphopeptide that was determined by the SEQUEST program was also manually inspected to ensure confidence.

Cell culture, transfection and lentiviral infection. LNCaP, PC-3, DU145, Rf and C4-2 prostate cancer cells were cultured in RPMI 1640 medium (Mediatech) containing 10% fetal bovine serum (FBS) (Hyclone). The prostate cancer cell line 22Rv1 and the immortalized prostate epithelial cell line BPH1 were cultured in RPMI 1640 medium supplemented with 5% FBS. The prostate cancer cell line LAPC-4 was grown in IMEM supplemented with 15% FBS. BJ, 293T and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS. RWPE-1 cells were cultured in keratinocyte serum-free medium supplied with recombinant human EGF (Invitrogen). Transfections were performed by electroporation using an Electro Square Porator ECM 830 (BTX) or by using Lipofectamine 2000 (Invitrogen). Approximately 75–90% transfection efficiencies were routinely achieved. Lentiviral infection was performed as described[35]. Roscovitine (30 μM, 12 h) was used to inhibit CDK.

RNA interference. siRNAs against the human *EZH2*, *CDK1* and *CDK2* genes and nonspecific control siRNAs were purchased from Dharmacon. The targeting sequences of four *EZH2* siRNAs and *CDK1* and *CDK2* siRNAs are listed in Supplementary Information, Table S1. siRNA-resistant (SR) wild-type and Thr 350A-mutated EZH2 were generated by site-specific mutagenesis (Stratagene).

Oligonucleotide microarray. LNCaP cells were transfected with EZH2-specific siRNAs and plasmids encoding siRNA-resistant wild-type EZH2 (EZH2^{SR}) and Thr 350A (Thr 350A^{SR}) mutant and incubated for 60 h or treated with DMSO, roscovitine (10 μM) or TSA (5 μM) for 24 h and then harvested for RNA isolation. Total RNA (2 μg) from all samples was prepared using the RNeasy Mini Kit (Qiagen). Synthesized cDNAs were hybridized to Affymetrix Human Genome U133 plus 2.0 microarrays (Santa Clara) at the microarray facility at BioMedical Genomics Center at the University of Minnesota. The data were normalized and analysed by dChip Software[37]. For BJ cells, lentivirus vectors were used to express GFP (control) or siRNA-resistant wild-type EZH2 (EZH2^{SR}) or T350A (T350A^{SR}) mutant. At 96 h after transduction, EZH2-specific or control siRNAs were transfected into BJ cells for 60 h. Total RNA (2 μg) from all triplicate samples was prepared using the RNeasy Mini Kit (Qiagen). cDNA was synthesized and hybridized to HumanHT-12 v4 Expression BeadChip (Illumina) in the microarray facility at the University of Minnesota. Hierarchical clustering was performed using the Genesis software in both studies[38]. Microarray profiling data were deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/, accession numbers GSE20433 and GSE22427).

Real-time RT-PCR. Total RNA was isolated from cells and cDNA was synthesized using the Super-Script kit from Invitrogen. Two-step real-time polymerase chain reaction (PCR) was performed using the SYBR Green Mix (BioRad) and an iCycler iQTM detection system (BioRad) according to manufacturer's instructions. Both forward and reverse primers were used at a final concentration of 200 nM. The primer sequences used for PCR are described in Supplementary Information, Table S2. The expression of *GAPDH* gene in each sample was used as an internal control.

***In vitro* HMTase assay.** The HMTase assay was modified from the method published previously[2,4,18]. Immunoprecipitated proteins from transfected 293T cells, or proteins expressed and purified from insect Sf9 cells and HeLa polynucleosomes, were added to the HMTase reaction buffer (12 mM Hepes at pH 7.9, 0.24 mM EDTA, 12% glycerol, 4 mM DTT, 2.5 mM $MgCl_2$, 0.5–1 μM ^{3}H-SAM, and 60–110 mM KCl), and then incubated for 60 min at 30 °C. Reactions were stopped with SDS-sample buffer, resolved by 18% SDS–PAGE, and transferred to Immobilon-P (Millipore), sprayed with EN^{3}HANCE (NEN) and exposed to film. The immunoprecipitated wild-type and mutated EZH2 proteins were analysed by western blot analysis with an anti-EZH2 antibody, and the insect expressed recombinant PRC2 complexes were visualised by Coomassie blue staining.

Chromatin immunoprecipitation (ChIP) assay. LNCaP cells were transfected with control siRNA, EZH2 siRNA or EZH2 siRNA plus siRNA-resistant wild-type EZH2 or Thr 350A mutant. At 60 h after transfection, cells were crosslinked with 1% formaldehyde and subjected to ChIP assay, as previously described[33]. The ChIP DNA was extracted with a PCR purification kit (Invitrogen) and was subjected to real-time PCR amplification using the primers specific for the H3K27me3 region in the promoters of *HOXA9* and *DAB2IP* genes (Supplementary Information, Table S2). The data for the occupation of H3K27me3 are expressed as a ratio of the cycle threshold for the chromatin immunoprecipitation DNA versus the cycle threshold for the input (1%) samples and further normalized to the values in transfected control cells.

Cell proliferation assay. Cell growth was monitored by absorbance using the MTS assay according to manufacturer's instructions (Promega). Briefly, cells were plated in 96-well plates at a density of 800 cells per well. At the indicated times, 20 μl of CellTiter 96R AQ_{ueous} Solution Reagent (Promega) was added to cells, and incubated for 2 h at 37 °C in the cell incubator. Cell growth was measured in a microplate reader at 490 nm.

DOI: 10.1038/ncb2116

Soft-agar colony formation assay. 22Rv1 cells were infected with lentivirus containing GFP (control) or wild-type or T350A-mutated EZH2 (LV-EZH2 or LV-T350A, respectively). At 3 days after infection, 10,000 cells were suspended in 2 ml RPMI 1640 medium containing 10% FBS and 0.3% agarose, and were then plated over a layer of solidified RPMI 1640 medium containing 10% FBS and 1% agarose in 6-well plates. When the agarose-containing cells were solidified, 2 ml of RPMI 1640 medium containing 10% FBS was added to cover the agarose. Plates were incubated at 37 °C and the medium was changed every 2 to 3 days for 2 weeks before colonies were photographed and counted.

Wound healing assay. RWPE-1 cells transiently transfected with EZH2, EZH2^{T350A} and control vectors were grown to confluency. Artificial wounds were created on the cell monolayer. Migrated cells and wound healing were visualised at 0, 24, 48 and 72 h.

Tumour specimens, immunofluorescent chemistry and confocal microscopy. Twelve cases of prostate tumours were selected from the surgical pathology files at the University of Minnesota Hospital (Fairview) from patients who had undergone radical prostatectomy between 1996 and 2008. The distribution of Gleason scores was as follows: Gleason 7 (eight cases), Gleason 8 (two cases), and Gleason 9 (two cases). This study was approved by the Institutional Review Board of the University of Minnesota. Sections (5 μm) were cut from formalin-fixed paraffin-embedded tissues. Tissues were deparaffinised and rehydrated, followed by 0.3% H_2O_2 treatment and several washes with 1 × PBS. Slides were subjected to immunofluorescent chemistry as previously described[22]. Cells or tissue sections were analysed with a laser-scanning microscope LSM510.

Statistics. Experiments were carried out with three or more replicates. Statistical analyses were performed by Student's *t*-test. Values with $P < 0.05$ are considered significant.

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DOI: 10.1038/ncb2116

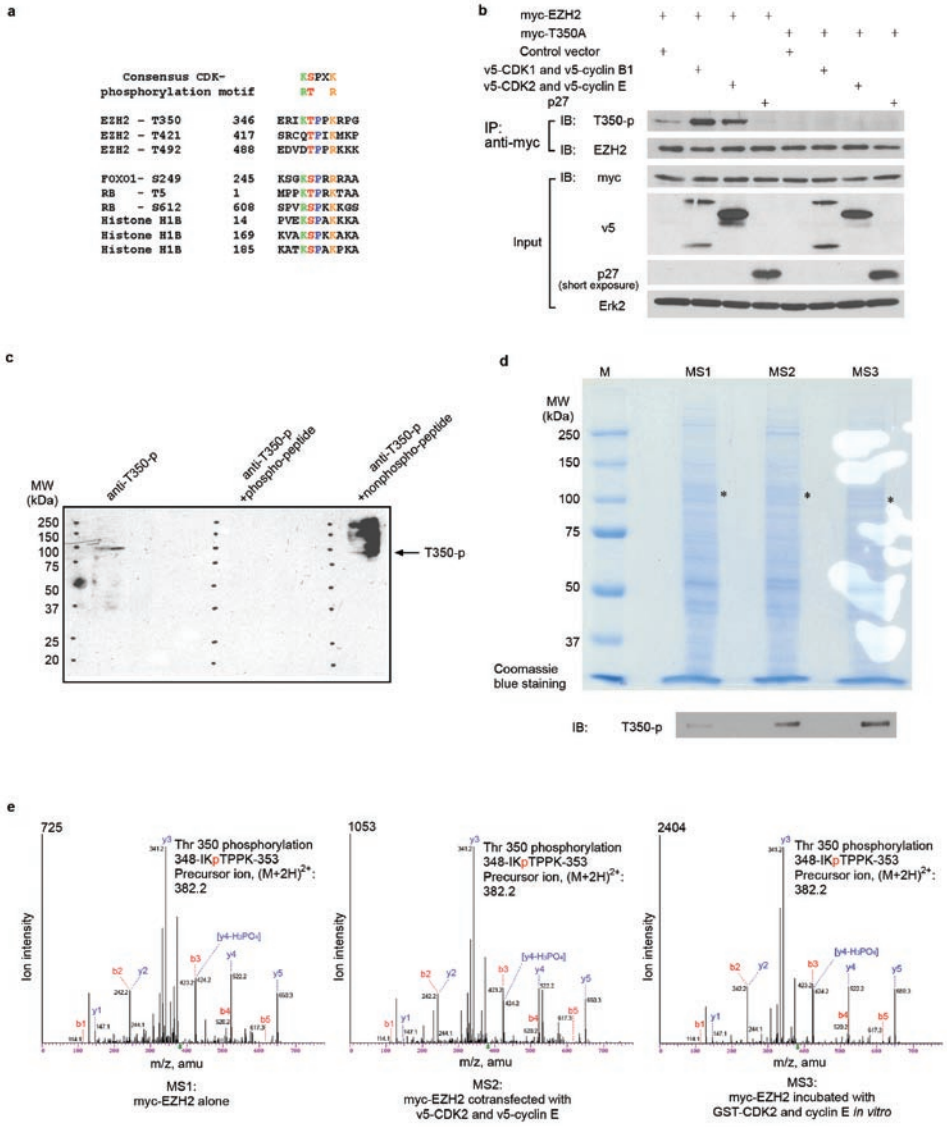

Figure S1 CDK phosphorylation of EZH2 in cells. (**a**) Comparison of candidate CDK phosphorylation sites on EZH2 with known CDK substrates by multiple sequence alignments. (**b**) CDK1 and CDK2 phosphorylates EZH2 at T350 *in vivo*. LNCaP cells were transfected with wild-type myc-tagged EZH2 or T350A mutant in combination with or without CDK1-cyclin B1, CDK2-cyclin E, or $p27^{KIP1}$. Ectopically expressed EZH2 proteins were immunoprecipitated with an anti-myc antibody and blotted with antibodies against the phosphorylated EZH2 at T350, myc-tagged EZH2 and proteins as indicated. Immunoblotting of Erk2 was included as a loading control. (**c**) Specificity of the antibody (T350-p) generated against the peptide containing the T350 phosphorylation site. LNCaP cell lysates were analyzed by SDS-PAGE and western blotting with anti-T350-p antibody, anti-T350-p antibody preblocked with the peptide containing the phosphorylated (AERIKpTPPKRPGC) or non-phosphorylated (AERIKTPPKRPGC) T350. (**d**, **e**) Mass spectrometry analysis of EZH2 T350 phosphorylation. 293T cells were transfected with myc-EZH2 (for samples MS1, MS2 and MS3) and v5-CDK2 and v5-cyclin E plasmids (for sample MS2). At 24 h after transfection, cell lysates were used for immunoprecipitation with anti-myc antibodies. For the precipitated myc-EZH2 in sample MS3, *in vitro* kinase assay was performed using bacterially produced and purified GST-CDK2 and GST-cyclin E. Protein samples were analysed by SDS-PAGE and Coomassie blue staining or western blot with anti-T350-p antibody (**d**). The bands around 110 kDa (indicated by * in (**d**)) were excised and subjected to in-gel tryptic digestion and micro-capillary LC-MS-MS mass spectrometry analysis. The MS-MS spectrum of the double charged ion (m/z 382.2) shows that the T350 residue is phosphorylated (low case p in red) in the peptide 348-IKTPPK-353 (**e**). The b ions (b1-b5) are the fragmentation ions containing the N-terminus of the peptide while the y ions (y1-y5) are the fragmentation ions containing the C-terminus of the peptide. The peak intensity values for IKpTPPK phosphopeptides in the samples MS1, MS2 and MS3 were 5.02E4, 6.11E4, and 1.35E5, respectively.

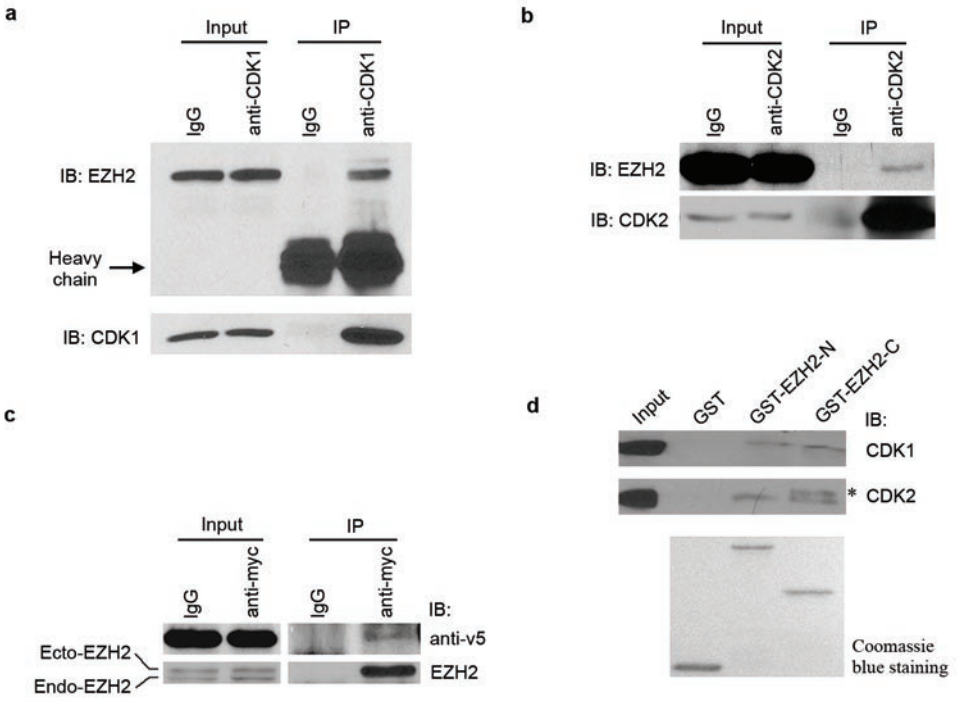

Figure S2 CDK1 and CDK2 form protein complexes with EZH2. (**a**) Co-immunoprecipitation of endogenous EZH2 and CDK1 proteins by an anti-CDK1 antibody in LNCaP cells. (**b**) Co-immunoprecipitation of endogenous EZH2 and CDK2 proteins by an anti-CDK2 antibody in LNCaP cells. (**c**) Co-immunoprecipitation of ectopically expressed v5-tagged CDK1 and myc-tagged EZH2 by an anti-myc antibody in LNCaP cells. (**d**) Whole cell lysates of LNCaP were subjected to GST pull-down assay with bacterially produced and purified GST, GST-EZH2-N (1-559), and GST-EZH2-C (560-741). CDK1 and CDK2 were immunoblotted by the corresponding antibodies. The asterisk indicates a nonspecific protein recognised by the anti-CDK2 antibody in the sample pulled down by GST-EZH2-C.

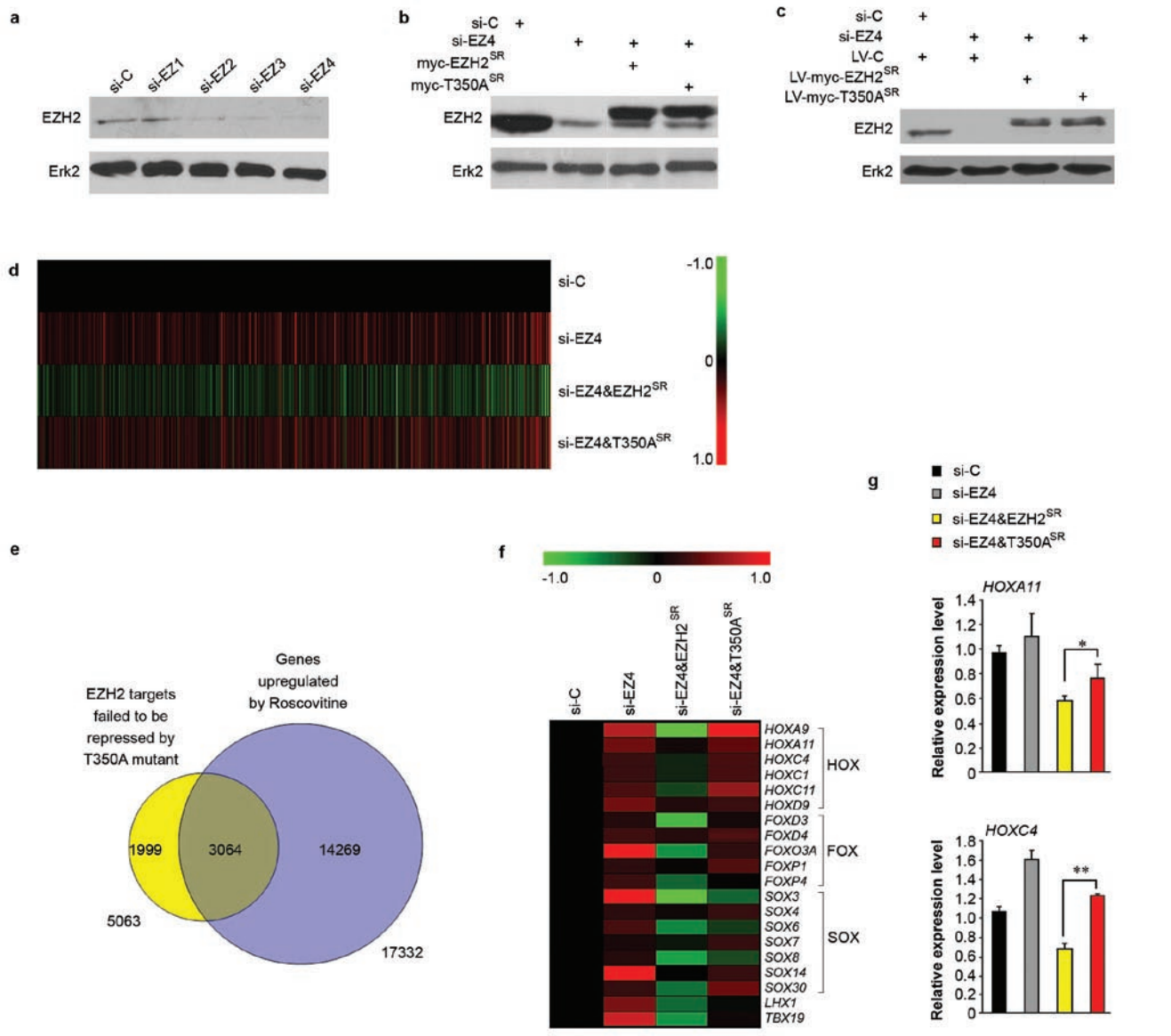

Figure S3 The effect of T350 phosphorylation on EZH2 target gene expression. (**a**) Efficiency of EZH2 knockdown by different EZH2 siRNAs. LNCaP cells were transfected with EZH2-specific siRNAs targeting four different sequences (si-EZ1, si-EZ2, si-EZ3, and si-EZ4) of the human EZH2 gene (targeting sequences of these siRNAs can be seen in Supplementary Information, Table S1). At 40 h after transfection, cells were harvested and cell lysates were subjected to western blot analysis with an anti-EZH2 antibody. si-EZ4 was chosen for subsequent experiments. (**b**) Knockdown of endogenous EZH2 and restored expression of wild-type and T350A-mutated EZH2. LNCaP cells were co-transfected with si-EZ4 with or without siRNA-resistant EZH2 plasmids as indicated. At 60 h after transfection, cells were harvested for analyses of western blot, or oligo microarray (Fig. 3a), ChIP assays (Fig. 4a and 4b) and real-time RT-PCR (Fig. 3b). (**c, d**) The effect of T350 phosphorylation of EZH2 on its repression of downstream target genes in normal human BJ fibroblasts. BJ cells were transducted with lentivirus containing GFP (control, LV-C) or wild type or T350A mutated EZH2 (LV-EZH2 or LV-T350A, respectively) for 96 h. Then cells were transfected with control siRNA (si-C) or EZH2 siRNA (si-EZ4) by electroporation. At 60 h after transfection, cells were harvested for western blot analyses (**c**). Hierarchical clustering of genes (represented by 10,314 probe-sets) exhibiting expression differences among the conditions as indicated in (**d**). Red and green represent upregulation and downregulation, respectively, relative to the reference samples (si-C). The overall fold changes in expression of EZH2 target genes mediated by EZH2 knockdown in BJ cells are lower than those in LNCaP cells (Fig. 3a), which is consistent with previous reports that EZH2 activity is much higher in cancerous cells than in normal cells[1]. (**e**) A Venn diagram shows the overlap between EZH2 targets that failed to be repressed by T350A mutant (Fig. 3a, lane 4) and all the genes upregulated by roscovitine in LNCaP cells. (**f**, **g**) Effects of T350 phosphorylation on the expression of development regulatory genes in HOX, FOX, SOX, LHX and TBX families in LNCaP cells. * $P<0.05$, ** $P<0.01$ (n = 3).

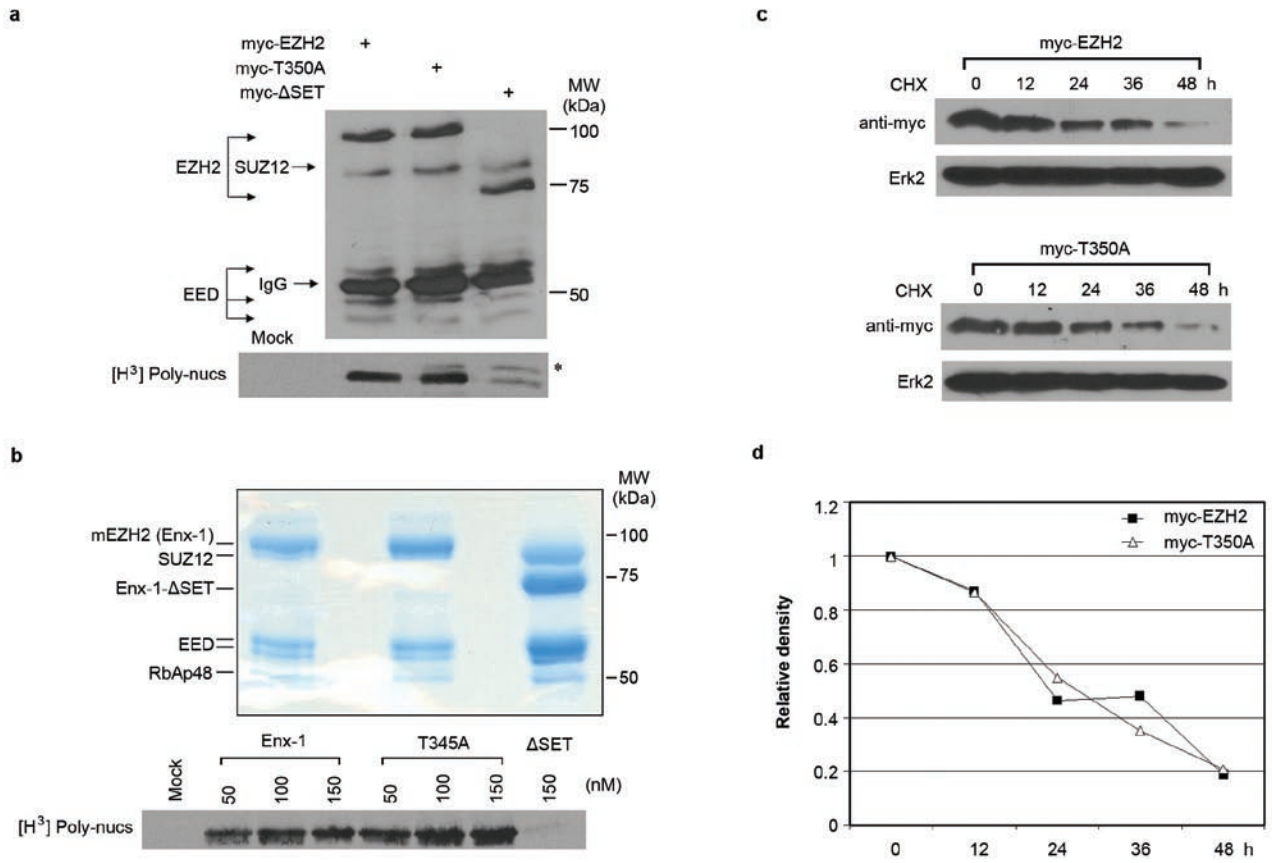

Figure S4 Effects of T350 phosphorylation on *in vitro* HMTase activity and protein stability of EZH2. (**a**) myc-tagged EZH2, T350A, or ΔSET mutants were overexpressed in LNCaP cells, and immunoprecipitated by anti-myc antibodies. The recovery of these proteins and SUZ12 and EED binding to EZH2 were evaluated by western blots. The methyltransferase activities of EZH2 were then tested by *in vitro* HMTase assay using HeLa polynucleosomes (poly-nucs) as substrate. The asterisk (*) indicates nonspecific bands. (**b**) Baculovirus vectors for wild-type or a phosphorylation-resistant mutant of mouse Ezh2 (Enx-1), in which the CDK site T345 (Fig. 1d) was mutated to alanine, were coinfected with the other three PRC2 components (Flag-EED, SUZ12, RbAp48) into insect Sf9 cells. PRC2 complexes containing different forms of Enx-1 were immunopurified by anti-Flag antibody and then subjected to *in vitro* HMTase assays using HeLa polynucleosomes as substrate. (**c**, **d**) Measurement of the half-life of wild-type and T350A mutant EZH2 proteins. LNCaP cells were transfected with myc-EZH2 or T350A mutant plasmids. At 24 h after transfection cells were treated with cycloheximide (20 μg ml^{-1}). Cells were harvested at the indicated time points and lysed for western blot analysis.

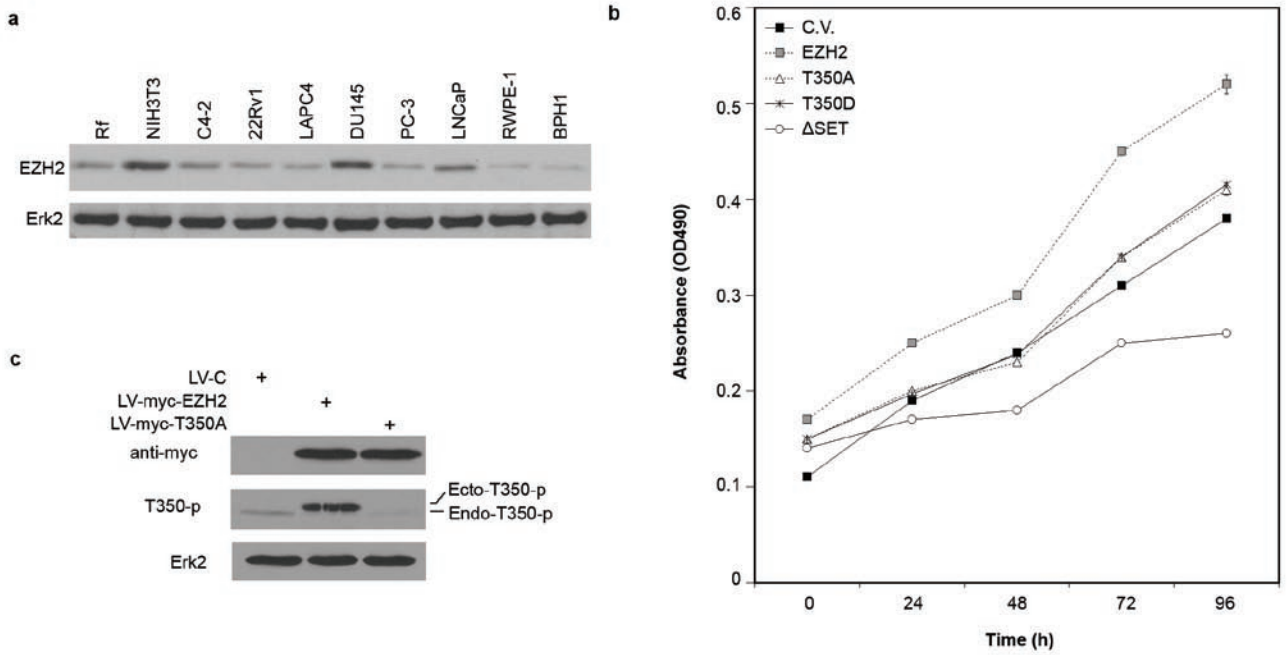

Figure S5 The effect of T350 phosphorylation on prostate cancer cell growth. (**a**) Western blot analysis of expression of the EZH2 protein in various cell lines, including prostate cancer cell lines Rf, C4-2, 22Rv1, LAPC4, DU145, PC-3 and LNCaP, immortalized prostate epithelial cell lines RWPE-1 and BPH1 and the NIH3T3 fibroblast cell line. Erk2 was used as a loading control. (**b**) Effects of EZH2 T350 phosphorylation on proliferation of PC-3 cells. PC-3 cells were transfected with the control vector (C.V.), wild-type EZH2 (EZH2), Thr-to-Ala (T350A) and Thr-to-Asp (T350D) mutants or catalytic-dead mutant (ΔSET). MTS assays were performed to examine cell proliferation at the indicated time points. (**c**) Evaluation of expression of wild-type and T350A mutated EZH2 proteins in lentivirus-infected 22Rv1 cells. 22Rv1 cells were infected with lentivirus containing the control vector (GFP, LV-C) and vectors for myc-tagged wild-type and T350A mutated EZH2. At 96 h after transfection, cells were harvested for western blot analyses with antibodies as indicated. Erk2 was used as a loading control. An additional set of cells were further carried out for soft agar colony formation assay as shown in Fig.5b.

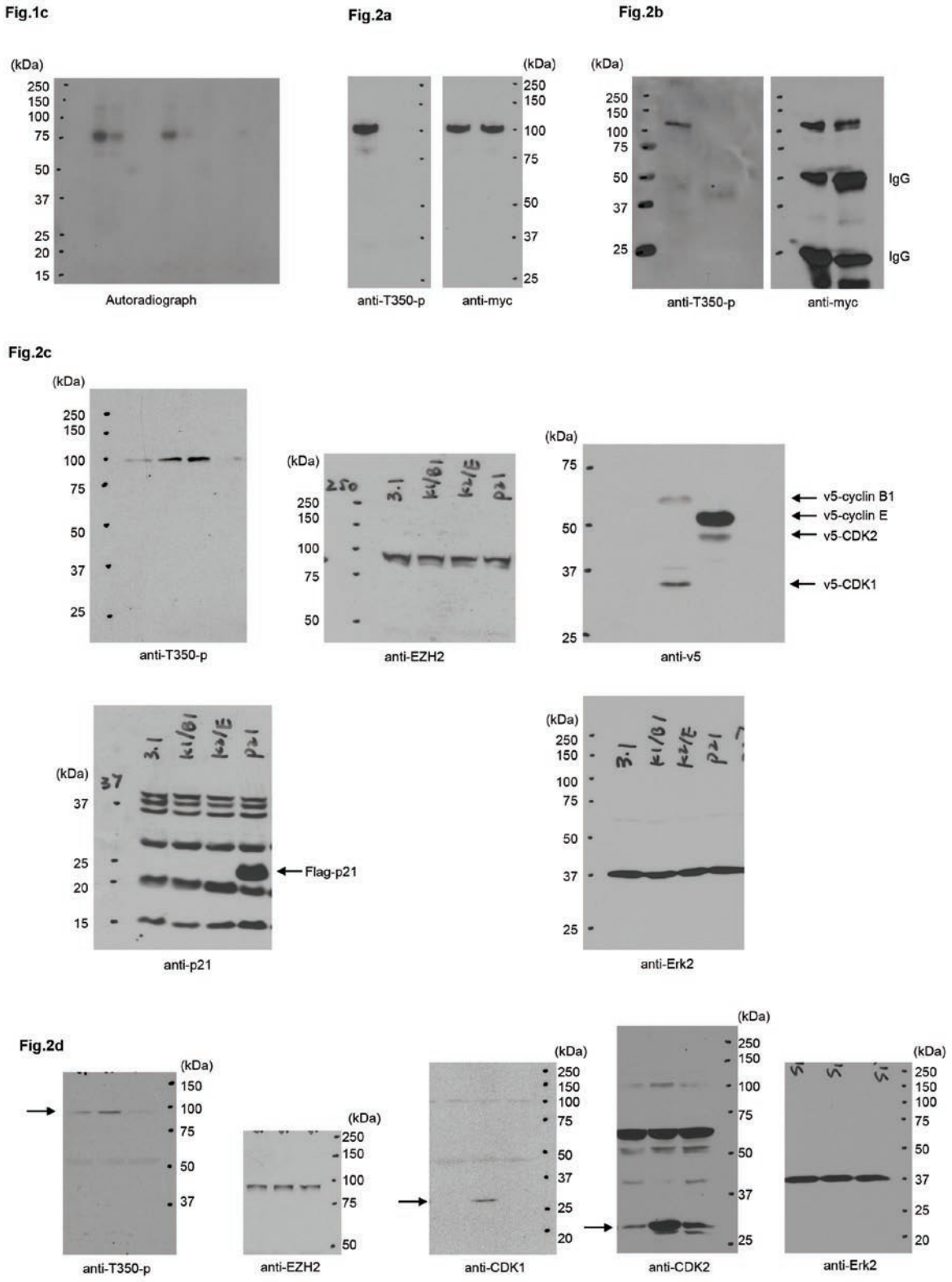

Figure S6 Uncropped version of the western blots presented in the main figures.

Fig.3c

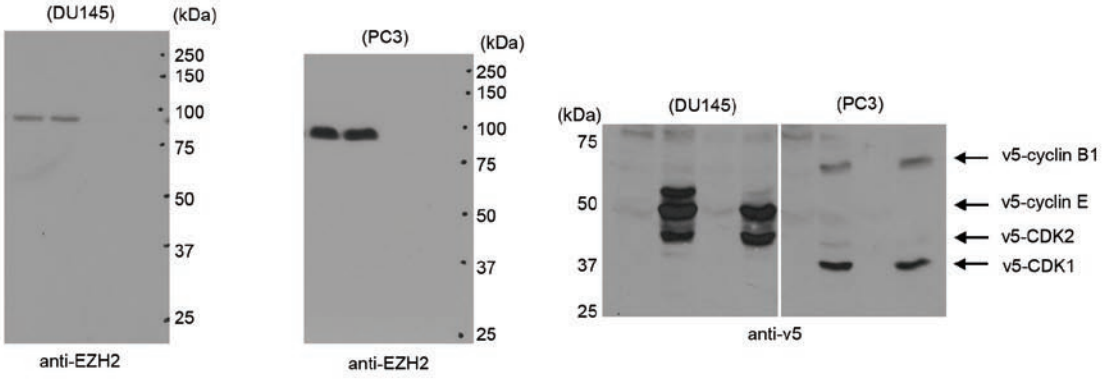

Fig.3d

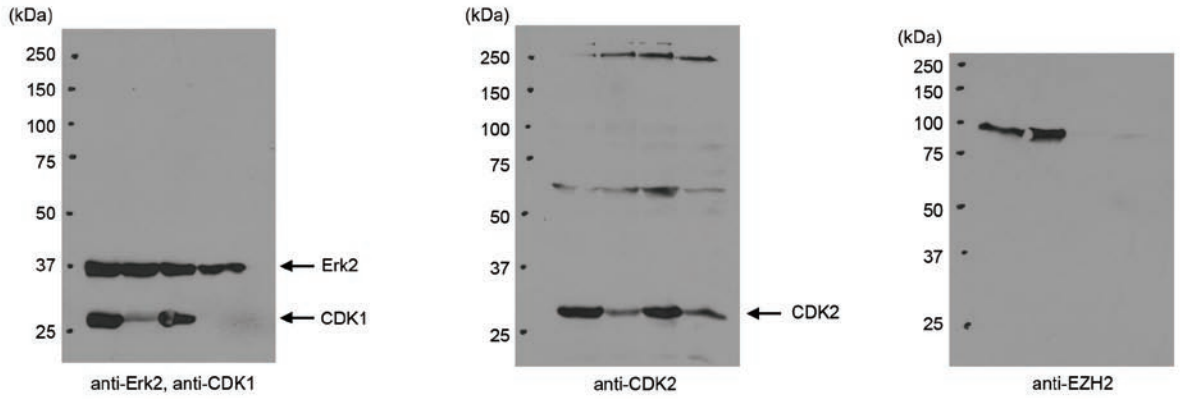

Fig.4

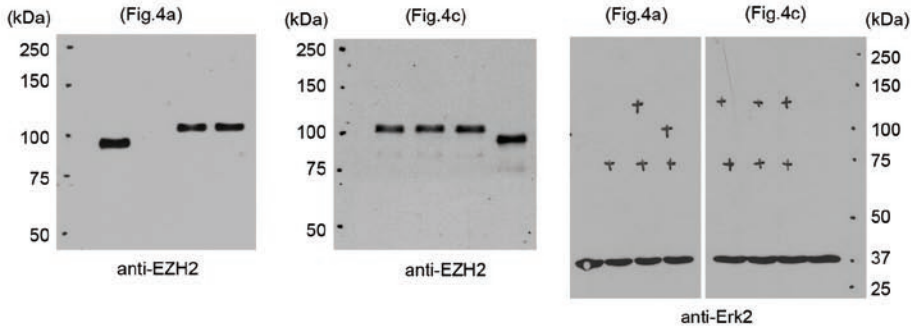

Fig.5a

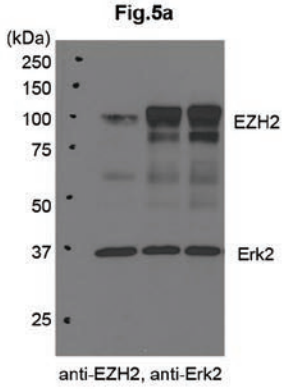

Figure S6 continued

SUPPLEMENTARY TABLES

Supplementary Table S1. Targeting sequences of siRNAs

EZH2 siRNAs	Targeting sequences
si-EZ1	CAAAGAAUCUAGCAUCAUA
si-EZ2	GAGGACGGCUUCCCAAUAA
si-EZ3	GCUGAAGCCUCAAUGUUUA
si-EZ4	GAAUGGAAACAGCGAAGGA
CDK1 siRNA pool	
Target1	GUACAGAUCUCCAGAAGUA
Target2	GAUCAACUCUUCAGGAUUU
Target3	GGUUAUAUCUCAUCUUUGA
Target4	GAACUUCGUCAUCCAAAUA
CDK2 siRNA pool	
Target1	GAGCUUAACCAUCCUAAUA
Target2	GAGAGGUGGUGGCGCUUAA
Target3	GCACCAAGAUCUCAAGAAA
Target4	GGACGGAGCUUGUUAUCGC

Supplementary Table S2. Primers for real-time RT-PCR and ChIP

Gene Names	Primers
(For Real-time RT-PCR)	
DAB2IP	5’-TGGACGATGTGCTCTATGCC-3’
	5’-GGATGGTGATGGTTTGGTAG-3’
HOXA9	5’-TTGGAGGAAATGAATGCTGA-3’
	5’-TGGTCAGTAGGCCTTGAGGT-3’
HOXA11	5’-CCATTGAATCTCCTTTGCCT-3’
	5’-CACACACGGTGGGTAAGAAC-3’
HOXC4	5’-TCCCTCCCACTGTTAAGGAC-3’
	5’-GAAATTCACCCAAGCCAGAC-3’
GAPDH	5’-GAAGGTGAAGGTCGGAGTC-3’
	5’-GAAGATGGTGATGGGATTTC-3’
(For ChIP assay)	
HOXA9	5’-TCGCCAACCAAACACAACAGTC-3’
	5’-AAAGGGATCGTGCCGCTCTAC-3’
DAB2IP	5’-CCTGCTTTCTGTTTCCTTCTCCTG-3’
	5’-TTGAACCACCTCCTCCTCCCTCTC-3’

REFERENCES FOR SUPPLEMENTARY INFORMATION

1. Varambally, S. *et al.* The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* **419**, 624-629 (2002).